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FULL PAPER

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Extraction Yield, Chemical Composition, Preliminary Toxicity of *Bignonia nocturna* (Bignoniaceae) Essential Oil and *in Silico* Evaluation of the Interaction

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Bignonia nocturna (Bignoniaceae) is a plant used for medicinal purposes by the Amazonian indigenous peoples. To date, there have been no reported studies on its toxicity. The present study aimed to evaluate the chemical composition of essential oils obtained from *Bignonia nocturna* by different extraction techniques. In addition, an *in silico* study of the molecular interactions was performed using molecular docking and molecular dynamics. The extractions were carried out by hydrodistillation, simultaneous distillation-extraction, and steam distillation, using samples collected from the Amazon in summer and winter. The chemical composition was analyzed by GC/FID and GC/MS, and the cytotoxic activity in *Artemia salina* Leach was evaluated. The maximum yield (1.38% w/w) was obtained by hydrodistillation. The results indicated that benzaldehyde predominated in all the fractions of both the volatile concentrate and the essential oils. In addition, the oil proved to be highly toxic to *Artemia salina*. The computer simulation results indicated that benzaldehyde strongly interacts with acetylcholinesterase, which is the likely interaction mechanism responsible for the cytotoxicity.

Keywords: amazon, natural products, *Tanaecium nocturnum*, cytotoxicity, acetylcholinesterase.

Introduction

In plants, essential oils (EOs) play an important role in terms of protection and communication, and in the resistance of phytopathogens and herbivores.[1] In industry, these oils are widely studied, mostly for their potential applications as promoters of biological activities.[2–5]

Essential oils can be extracted by various techniques, depending on their intended use and on the plant organ and quantity of plant material available.[6–9] One of the most noteworthy methodologies used to obtain essential oils is hydrodistillation (HD), which is considered one of the simplest techniques. This technique is used on a laboratory scale, and is a replica of the method used in industry, in which the essential oil obtained has similar characteristics.[9,10]

Another important method is steam distillation (SD), which follows the same principles as HD. This technique is widely used in industry for the production of essential oils for commercialization. It is considered a simple and inexpensive process, and can be applied on laboratory, pilot, and industrial scales, thus enabling the processing of significant amounts of botanical material.[11–14] The simultaneous distillation-extraction (SDE) method, which uses the Likens-Nickerson apparatus, is another conventional extraction method used to obtain volatile constituents, including aromas and fragrances.[15–17] This technique is superior to other traditional extraction methods in terms of obtaining volatiles, because it is reproducible,

highly efficient, and requires only a small amount of sample.[16,17] This justifies its frequent use for quality control.

Bignonia nocturna (Barb.Rodr.) L.G.Lohmann (= *Tanaecium nocturnum* (Barb.Rodr.) Bureau & K.Shum.) of the Bignoniaceae family is one among several Amazonian species that produce essential oil. Known as cipó vick or cipó-corimbó, it is a climbing plant with a strong smell, characteristic of bitter almonds. Its leaves are used externally by the local population as an analgesic. The indigenous people of the Gotira village, located on the banks of the Fresco River, a tributary of the Xingu River, and the members of the Kayapós tribe (Pará, Brazil) use the plant stems as insecticides, and as decongestants for the airways.[18–20] In addition, biological tests, such as toxicity tests, have been designed to assess or predict toxic effects on biological systems and to measure the relative toxicity of substances.[21,22] These assays also serve as the first step in cytotoxicity screening, which can direct the development of new drugs.[23]

The present study was aimed to analyze the extraction yield, chemical profile, preliminary cytotoxicity, and carry out an *in silico* study of the interaction mechanism of the majority component with a target protein in *Artemia salina* Leach.

Results and Discussion

Extraction Yield

Table 1 shows the extraction yields of essential oils obtained by HD and SD. The HD process was the best technique for the extraction of fresh leaf (FL) samples (yield = 1.13% w/w dry basis). The best yields for dry leaf (DL) and stem samples (0.31% and 0.35%, respectively; dry basis) were obtained by SD and HD, respectively. In general, extraction by HD was more efficient than that by SD. HD was also found to be a more efficient process for extracting essential oils from *Platycladus orientalis*,[24] and *Origanum majorana*.[25] However, for *Rosmarinus officinalis*,[26] and *Thymus vulgaris*,[27] HD was not the most efficient method. This demonstrates that in addition to the extraction technique, the type of raw material used may also influence the yield of essential oil. Pimentel et al.[28] reported an essential oil content of 1.55% in fresh leaves (62% moisture) of *Bignonia nocturna* [cited as *Tanaecium nocturnum*], higher than that found in this study.

Chemical Composition

The different essential oil fractions and aromas of *Bignonia nocturna* obtained by HD, SDE, and SD are shown in *Table 2*. The 18 compounds identified account for ~99% of the chemical composition, and are formed mostly via the metabolic route of benzenoids. Benzaldehyde was the predominant substance identified in all parts of the plant and in all oil fractions and aromas analyzed. The highest concentration was obtained by SDE of the aroma fraction of fresh leaves (97.24%). In addition, benzoic acid was identified at concentrations of 11.33% and 4.75% in the aroma fractions obtained from fresh stem (FS) and dry stem (DS) samples, respectively. When fresh leaves were processed using HD, the benzoic acid concentration obtained was 0.21%, while SD of fresh stems afforded 0.39%.

Benzaldehyde was the major constituent in the essential oil fractions from fresh stems obtained by HD and SD, yielding 99.5% and 98.62%, respectively. SD of the essential oils afforded the best benzyl benzoate yield (6.07%) when using fresh leaves, while HD and SDE afforded the highest levels of benzyl benzoate (0.79–2.5%) when using dry material (leaves and stems). No volatiles were obtained from the fresh stems. There have been other reports on benzaldehyde being the major compound in the *Bignonia nocturna* [cited as *Tanaecium nocturnum*] essential oil.[29,30]

Principal component analysis (PCA) (*Figure 1*) and hierarchical cluster analysis (HCA) (*Figure 2*) were applied to the essential oil constituents from the leaves and stems of *Bignonia nocturna*, which afforded yields ≥ 0.1%, to evaluate the similarities between essential oil samples obtained by each of the different extraction techniques. The two main components PC1 (35%) and PC2 (31.8%) had variances of 66.8%. The samples were divided into four groups (*Figure 1*).

Table 1. Extraction yields of *Bignonia nocturna* essential oils obtained by hydrodistillation and steam distillation.

	Hydrodistillation				Steam distillation			
	Fresh leaves	Fresh stems	Dry leaves	Dry stems	Fresh leaves	Fresh stems	Dry leaves	Dry stems
% EO (w/w)	1.13	0.35	0.92	0.18	0.28	0.28	0.31	0.05

Table 2. Chemical composition from fractions of *Bignonia nocturna* essential oils and aroma.

RI_L[a]	RI_C[b]	Constituents	Hydrodistillation (HD)				Simultaneous distillation-extraction (SDE)				Steam distillation (SD)			
			FL[e]	DL[f]	FS[g]	DS[h]	FL[e]	DL[f]	FS[g]	DS	FL[e]	DL[f]	FS[g]	DS[h]
846[c]	846	(2*E*)-Hexenal		0.16			0.14	0.52	0.46	0.5		0.19		
952[c]	952	Benzaldehyde	98.75	98.08	99.5	96.67	97.24	95.36	85.67	91.72	89.79	97.6	98.62	93.1
974[c]	978	1-Octen-3-ol	0.49		0.44	1.15	0.6	0.42	0.75	0.74	0.47	0.22	0.65	1.58
979[c]	984	3-Octanone								0.05				
988[c]	992	3-Octanol							0.16	0.16				
1026[c]	1026	Benzyl alcohol		0.07	0.02	0.24	0.06	0.22	0.32	0.9	0.4	0.48	0.21	1.13
1036[c]	1036	Benzeneacetaldehyde									0.02			0.02
1071[c]	1071	Benzyl formate				0.03		0.03			0.03	0.02		0.09
1088[c]	1088	Methyl benzoate									0.05			0.02
1169[c]	1175	Ethyl benzoate				0.09								
1160[d]	1185	Benzoic acid	0.21					0.25	11.33	4.75	0.05		0.39	0.24
1190[c]	1190	Methyl salicylate	0.03	0.02		0.13	0.02	0.07		0.2	0.11	0.03	0.02	0.44
1356[c]	1356	Eugenol						0.03						0.06
1403[c]	1405	Methyl eugenol									0.59	0.02		0.08
1565[c]	1559	(3*Z*)-Hexenyl benzoate		0.04			0.04	0.12			0.33	0.09		
1579[c]	1579	Hexyl benzoate		0.01			0.02	0.07			0.04	0.04		
1587[c]	1590	(2*E*)-Hexenyl benzoate						0.03						
1759[c]	1759	Benzyl benzoate	0.07	1.22		1.29	1.22	2.5		0.46	6.07	1.09		1.59
Total			99.55	99.6	99.96	99.6	99.34	99.62	98.69	99.48	97.95	99.78	99.89	98.35

[a] RI_L: Literature Retention Index; [b] RI_C: Calculated Retention Index; [c] Adams[48]; [d] Nist (Stein et al.)[49]; [e] FL: Fresh leaves; [f] DL: Dry leaves [g] FS: Fresh stem; [h] DS: Dry stems.

Group I consisted of the samples FL-HD, DL-HD, FS-HD, DS-HD, DL-SD, FS-SD, DL-SDE, and FL-SDE. Samples were selected for this group based on their benzaldehyde content. Group II combined the DS-SDE and FS-SDE samples, for which (2*E*)-hexenal, 3-octanol and benzoic acid were the main components. Group III included the FL-SD sample, that contained the chemical compounds methyl eugenol, benzyl benzoate, and (3*Z*)-hexenyl benzoate. Finally, group IV included the DS-SD sample, which contained methyl salicylate, benzyl alcohol, and 1-octen-3-ol.

The groups formed during the PCA were confirmed by HCA and the results were expressed using a dendrogram (*Figure 2*), with four groups at different levels of similarity. The multivariate analysis of group I samples revealed several suitable options for obtaining essential oils from *Bignonia nocturna* with high levels of benzaldehyde (an important chemical compound used as an artificial food flavor). These include HD of any part (leaves or stems) of the fresh or dried plant, as well as SD of the dried leaves and fresh stems. In the same group, there are samples of fresh and dried leaves obtained by SDE that have the same chemical profile as other samples in the group. Since these last two refer to aroma, they could be used to obtain benzaldehyde, which is widely used in the food industry.

Preliminary Toxicity in Artemia salina

There was no mortality in the tests carried out with the control group, which meant that dimethyl sulfoxide (DMSO) was a viable solvent for the assays with *Artemia salina*. *Table 3* shows the results for

Table 3. Preliminary toxicity of *Bignonia nocturna* essential oil in *Artemia salina*.

Essential oil	Concentration (μg mL^{-1})	Mortality (%)	LC_{50} (μg mL^{-1})
	100	100	
Fresh leaves	50	100	
1.13	25	100	4.2 ± 3.3
	10	80	
	5	40	

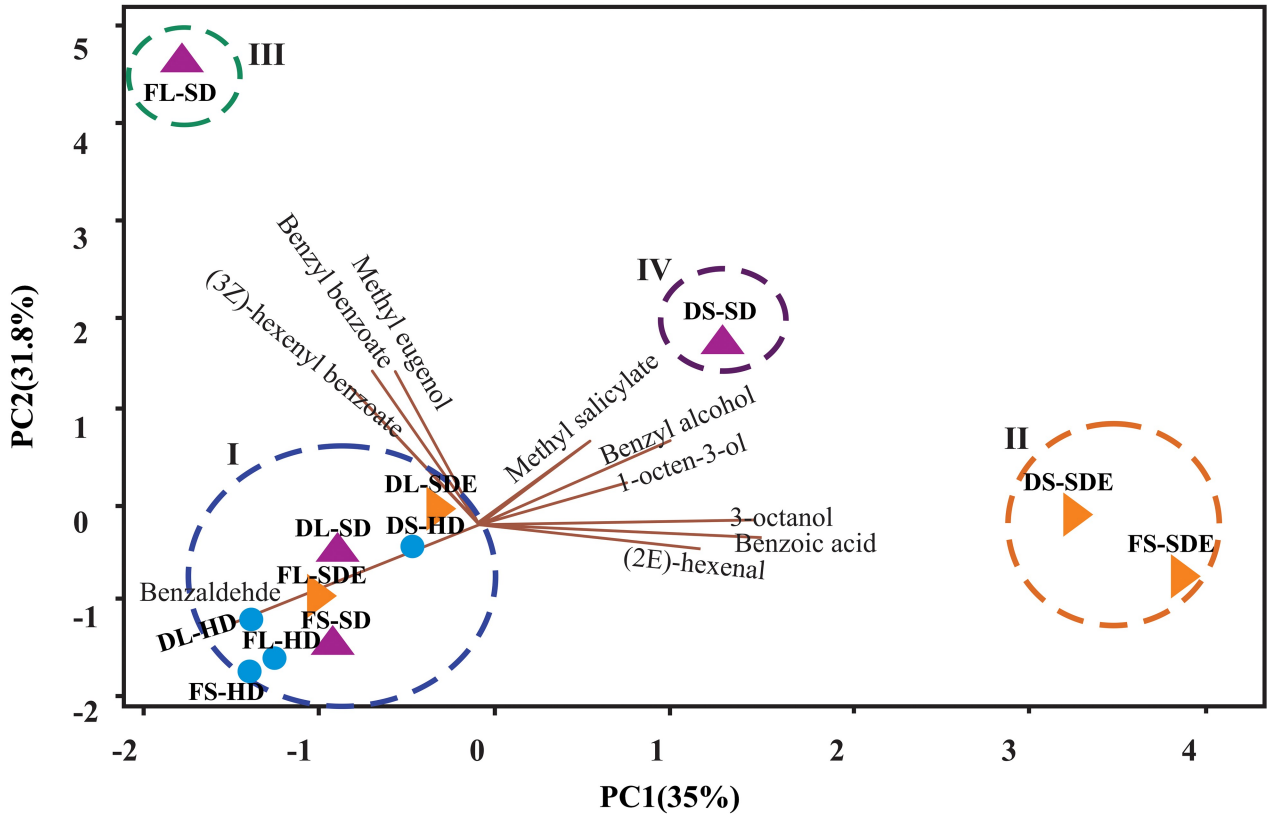

Figure 1. Biplot (PCA) resulting from the analysis of oil samples from leaves and stems of *Bignonia nocturna* obtained by different extraction methods. DH: Hydrodistillation. SD: steam distillation. SDE: Simultaneous distillation-extraction. FL: Fresh leaves. DL: Dry leaves. FS: Fresh stems. DS: Dry stems.

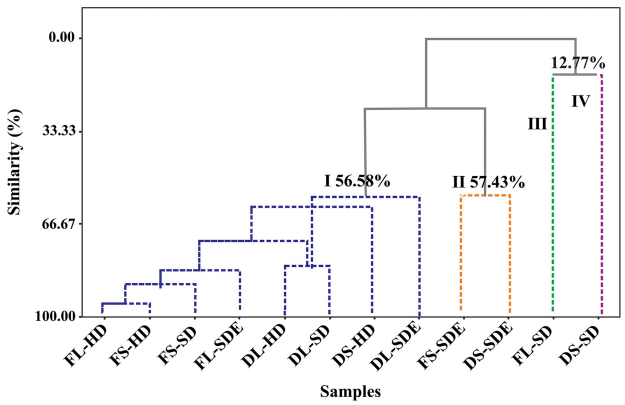

Figure 2. Dendrogram representing the relative similarities of essential oils samples from leaves and stems of *Bignonia nocturna* obtained by different extraction methods. DH: Hydrodistillation. SD: steam distillation. SDE: Simultaneous distillation-extraction. FL: Fresh leaves. DL: Dry leaves. FS: Fresh stems. DS: Dry stems.

different concentrations of *Bignonia nocturna* essential oil. The lethal concentration (LC_{50}) was calculated by converting the mortality percentage of larvae into products, which could be plotted against the logarithm of the concentration to obtain an equation. According to the literature,[31] samples with LC_{50} values below 30 $\mu g\,mL^{-1}$ are considered toxic. The oil proved to be highly toxic ($LC_{50} = 4.2 \pm 3.3\ \mu g\,mL^{-1}$), which indicates 12 times more activity than the lapachol cytotoxic standard ($LC_{50} = 22.0 \pm 1.9\ \mu g\,mL^{-1}$). Some examples of other essential oil samples, with different plant origins and different biological functions, are: *Citrus limon* ($LC_{50} = 743.35\ mg\,L^{-1}$),[32] *Hyptis suaveolens* ($LC_{50} = 49.72\ \mu g\,mL^{-1}$),[33] and *Syzygium aromaticum* ($LC_{50} = 0.59 \pm 0.04\ \mu g\,mL^{-1}$).[34] Low LC_{50} values in *Artemia salina* demonstrate that the extract is highly toxic and harmful to humans, and should therefore be administered with care to avoid poisoning.[33,35]

In Silico Analysis

In silico molecular docking simulations have been applied to investigate the interaction of bioactive compounds of natural origin with proteins of pharma-

cological interest.[36,37] We used this technique to investigate whether benzaldehyde, a major compound in essential oil from *Bignonia nocturna*, is capable of interacting with the acetylcholinesterase (AChE) protein. This protein has been reported in the literature as an important target related to *Artemia salina* mortality.[38–40]

To develop the docking methodology, we first attempted to reproduce the experimental connection mode observed in the AChE crystal, using our docking protocol, as described in the methodology section. Then, the PDB 4 M0E crystallographic ligand was redocked with AChE using the Molegro Virtual Docker software. Each redocking pose was evaluated by considering the root mean-square deviation (RMSD) values and docking scores. According to the literature, the RMSD values between the redocked protein and crystallographic ligand should be less than 2 Å.[41–43]

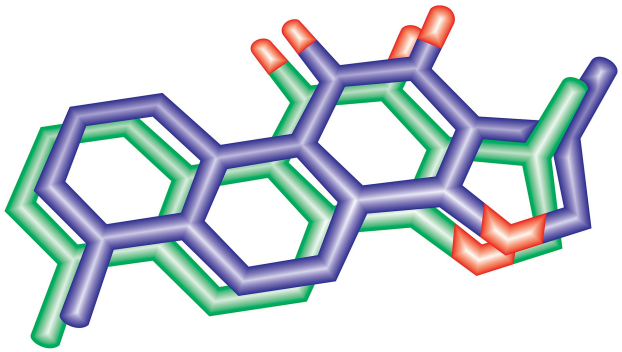

Figure 3. The structure obtained by redocking (blue), overlapping the crystallographic structure (green).

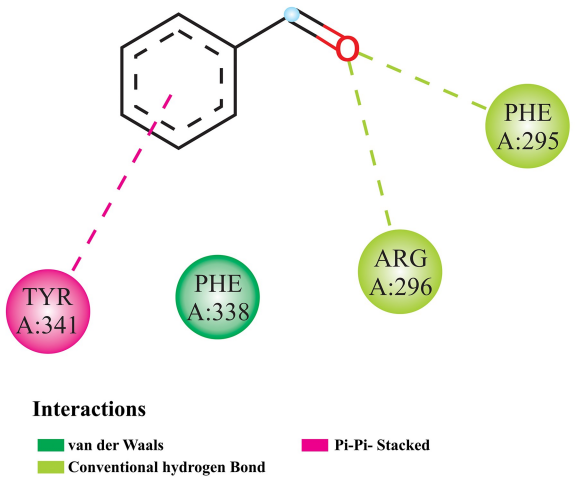

Figure 4. Molecular interactions established between benzaldehyde and the residue of the AChE active site.

We obtained an RMSD of 1.34 Å. *Figure 3* shows the overlap of the ligand structures. After our protocol had been optimized to predict an interaction mode observed experimentally, we repeated the experiment with the same parameters to evaluate the interaction of benzaldehyde with AChE.

The most favorable conformation of benzaldehyde docked with AChE is shown in *Figure 4*. The interaction energy obtained from the MolDock score was $-73.52\ \text{kcal mol}^{-1}$. In this conformation, the ligand interacted with important AChE binding pocket residues that are related to the inhibition of this protein. The interactions established between benzaldehyde and AChE are shown in *Figure 4*.

A π–π interaction was established with the Tyr341 residue, Van der Waals interactions were established with Phe338, and hydrogen bonds were formed with Arg296 and Phe295. After analyzing these interactions, molecular dynamics simulations were performed to assess the behavior of the system over time. In these simulations, benzaldehyde continued to interact with the AChE active site. Using the MM-GBSA method, we evaluated the interaction energy of this complex. The value obtained was $-32.75\ \text{kcal mol}^{-1}$, which demonstrates that the established system is favorable and contributes to enzyme inhibition.

Conclusions

Bignonia nocturna is an important source of essential oil with high benzaldehyde content. This plant has been used medicinally in the Amazon. The essential oil of *Bignonia nocturna*, obtained from the aerial parts of the plant, demonstrated high cytotoxicity against *Artemia salina*, suggesting that care must be taken to avoid poisoning when using this product. This result may also be related to the synergy between its chemical components, which indicates that more precautions should be taken regarding dosages and the frequent use of *Bignonia nocturna* essential oil. Benzaldehyde was found to interact with the active site of AChE via van der Waals forces and hydrogen bonds. Throughout the simulation, the ligand remained in the active site and this was reflected by a favorable affinity energy value.

Experimental Section

Botanical Material Collection

The aerial parts of the species *Bignonia nocturna* were collected from the Brazilian Agricultural Research Corporation (Embrapa) area in 2018, located in Belém, Pará, Brazil (01°0'28" S, 048°0'27" W, altitude: 12 m). Approximately 3 kg of plant material was collected between leaves and stems. This specimen was identified by comparison with an authentic specimen from the herbarium of the Museu Paraense Emilio Goeld, Belém, Pará, Brazil. Voucher (MG 216253).

Sample Preparation

In the laboratory, the fresh botanical material was separated into leaves and stems. Then, the material was divided into two portions (1 kg each). One portion was designated for extraction, and the other was placed in an air circulation oven at 34 °C for five days. The samples were ground, homogenized, weighed, and subjected to the extraction processes. It should be noted that the plant was cut into small pieces with scissors, homogenized, and weighed *in natura* before extraction.

Extraction Methods

Hydrodistillation

For the HD extraction process, dry and fresh sample (40 g each) were used. The same proportion of water in relation to plant material was used, according to the literature.[44,45]

Simultaneous Distillation-Extraction

For aroma extraction, 10 g each of dry and fresh sample was prepared, mixed with water (20 mL), and subjected to SDE for 3 h using a Likens-Nickerson Extractor with pentane (2 mL) as the organic mobile phase, as described in the literature.[46]

Steam Distillation

SD of dry and fresh samples was performed using a modified Clevenger-type glass system, coupled with a cooling system for maintaining condensation water at 10–15 °C for 3 h. After extraction, the obtained oils were centrifuged for 300 s at 3000 rpm and then dehydrated with anhydrous Na_2SO_4. The oil yield was calculated as a percentage (%). The oils were stored in amber glass ampoules, sealed with a flame, and stored at −15 °C.

Analysis of the Chemical Profile of Essential Oils and Aromas

Analyses of the chemical profiles of the aroma and essential oil fractions of from *Bignonia nocturna* were performed using protocols reported earlier by our research group.[45] The retention index (RI) was calculated in accordance with a different study.[47] Individual components were identified by comparing both mass spectra and RI, as well as with the aid of commercial libraries containing RIs and mass spectra.[48,49]

Preliminary Toxicity Analysis

For the toxicity tests, the sample with the highest mass yield was selected. The essential oil was prepared at concentrations of 100, 50, 20, 10, 5, and 1 $\mu g\,mL^{-1}$. Ten *Artemia salina* larvae were added to each test flask with the aid of automatic micropipettes. Brine water (artificial) and DMSO were used as solvents with a 95:5 ratio. In the control group and the positive group with lapachol, the same solvent was used for the samples and larvae under the same conditions as the bioassay. After 24 h of contact between the larvae and the sample solution, the dead larvae were counted (in each concentration), and the mortality rate and the IC_{50} value were calculated using the Probitos statistical method.[31,50] All the experiments were performed in triplicate ($n = 3$).

In Silico Molecular Target Prediction

It was used the molecular docking method for predicting benzaldehyde interaction mode with the active site of the enzyme acetylcholinesterase. Docking was performed with the Molegro Virtual Docker 5.5 software (MVD).[42,51,52] The AChE structure used as a molecular target can be located at the Protein Data Bank (http://www.rcsb.org), with the ID: 4 M0E.[53]

The molecular atomic charges were obtained with the Restrained Electrostatic Potential (RESP).[54,55] The parameters for each molecule were constructed using the Antechamber module, described by General Amber Force Field.[56] The modules sander and pmemd.CUDA from Amber16 package were used for the Molecular Dynamics Simulations.[57,58] The force field ff14SB,[59] was used for all MD simulations. The

systems were solvated in an octahedron periodic box with water TIP3P model.[60]

The free energy of each complex was obtained from the last 5 ns of the trajectory. The MM-GBSA approach, free binding energy is calculated from the free energy of a ligand interacting with a receptor to form the complex.[36,61,62]

Statistical Analysis

Multivariate analysis was performed according to a reported procedure,[63–65] using the Minitab® software. The variables used were the chemical compounds of the essential oil and aroma of *Bignonia nocturna* leaves and stems ($\geq$0.1 %) obtained by HD, SD and SDE, thus forming a matrix of 12 (samples)×10 (variables). The raw data were first standardized by weight.

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Author Contribution Statement

M.S.O. and V.M.P.S.: manuscript writing and research. L.C.F.: analysis. S.G.S. and J.N.C.: *in silico* study. E.H.A.A.: project coordinator and manuscript review.

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