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Chemical composition and antimicrobial activity of leaf and bark essential oils of *Apodocephala pauciflora* Baker (Asteraceae)

Noelinirina Raharisoa [1], Mihajasoa Stella Razanatseheno [1], Rahanira Ralambondrahety [2], Zara Nomentsoa Razafiarimanga [1], Lovarintsoa Judicael Randriamampianina [1], Hanitra Ranjana Randrianarivo [1], Danielle Aurore Doll Rakoto [1] and Victor Louis Jeannoda [1,*]

[1] *Laboratory of Applied Biochemistry to Medical Sciences, Fundamental and Applied Biochemistry Department, Faculty of Sciences, University of Antananarivo, P.O. Box 906, Antananarivo 101, Madagascar.*
[2] *National Environmental Research Centre (CNRE), P.O. Box 1739, Antananarivo 101, Madagascar.*

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Abstract

The present work aims to study the chemical composition and the antimicrobial and toxicological properties of the essential oils (EOs) of *Apodocephala pauciflora* leaves (LEO) and stem bark (BEO). LEO and BEO were extracted from fresh material by hydrodistillation with a yield of 0.1%. They are light, light yellow, strong smelling and dextrorotatory. Gas chromatography/mass spectrometry (GC/MS) analysis identified 42 components in LEO and 38 in BEO representing 97.54% and 99.44% of the overall composition respectively. In LEO, the major components were α-pinene (27.5%), sabinene (13.62%) and β-pinene (12.0%) and in BEO, α-pinene (34.32%), myrcene (15.1%), sabinene (14.53%). Main components such β-pinene, phellandrene and limonene were common to LEO and BEO but at different rates. However, some components were not common to both EOs: for example, cubenol (5.07%) in LEO was absent in BEO and vice versa humulene (3.91%) in BEO was absent in LEO. Both EOs were effective against all microorganisms tested, including Gram (+) and Gram (-) bacteria and a fungus, with a strain-dependent intensity. BEO was more efficient than LEO. *Streptococcus pneumoniae, Pseudomonas aeruginosa, Vibrio fischeri* and *Candida albicans* were the most susceptible. LEO was bacteriostatic against *Clostridium perfringens* and *Candida albicans* but bactericidal against the other germs tested, whereas BEO was bactericidal against all germs. With LD50 of 2.48 and 2.34 g/kg body weight, LEO and BEO were slightly toxic to mice by oral route. LEO and BEO could be used as alternatives to synthetic antibiotics against several pathogenic microorganisms.

Keywords: *Apodocephala pauciflora*; Essential Oil; Physico-chemical Properties; Antimicrobial Activity; Toxicity

1. Introduction

The growing interest in essential oils is reflected in the extensive research being carried out around the world on aromatic plants. They have many exploitable properties that allow them to be used in a wide variety of fields such as the pharmaceutical, food, flavour and fragrance, perfumery and cosmetics industries, as well as agriculture [1, 2].

In human medicine, they have an extremely wide range of activities (anti-infectious, anti-inflammatory, antihistaminic, immunoregulatory, vasculotropic, neurotropic, endocrinoregulatory, antitumour, etc. properties) [2].

* Corresponding author: Jeannoda Victor Louis
Laboratory of Applied Biochemistry to Medical Sciences, Fundamental and Applied Biochemistry Department, Faculty of Sciences, University of Antananarivo, P.O. Box 906, Antananarivo 101, Madagascar.

Faced with the increase in multi-drug resistant pathogens worldwide, the search for new molecules of natural origin is developing in many countries. Studies have shown that aromatic plants are a reservoir of new remedies [3, 4]. They are considered as sources of raw materials for the discovery of new molecules necessary for the development of future drugs [5]. Malagasy plants are good candidates given the richness and diversity of the flora whose originality could promise original molecules.

As part of our research on the antimicrobial properties of essential oils from plants endemic to Madagascar, we chose to study *Apodocephala pauciflora*, an aromatic plant of the Asteraceae family growing in the Mandraka forest.

The main objectives of this study were to determine the composition and physico-chemical characteristics of *Apodocephala pauciflora* leaf and stem bark EOs and to explore their potential antimicrobial and toxic activities.

2. Material and methods

2.1. Materials

2.1.1. Plant material

Apodocephala pauciflora Baker, known by its vernacular name "Tsiramiramy", is one of the 9 species of *Apodocephala* which is an endemic genus to Madagascar. It is a small to medium sized evergreen tree, reaching 10-30 m in height with a trunk of 60 cm in diameter and a bark with a whitish fragrant exudate (Figure 1).

Figure 1 *Apodocephala pauciflora*: Tree; leaves and inflorescence (Source: The authors)

Apodocephala pauciflora is distributed in the north and east of central Madagascar. Leaves and stem bark were collected in July 2021 in the Mandraka rainforest, located at 70 km east of Antananarivo with geographic coordinates 18° 53′ 7'' South, 47° 56′ 16.8'' East. At the time of harvest, the plant was in the vegetative stage (without flowers or fruits). It was identified at the Botanical and Zoological Park of Tsimbazaza by comparison with the herbarium n°1174 established by B. Lewis, F. Rasoavimbahoaka and J. Rastefanonirina in October 1994.

2.1.2. Microbial strains

The microbial strains used included 4 Gram (-) and 4 Gram (+) bacteria and one fungus (Table 1).

Table 1 List of microbial strains used

Germ-Tests	Gram	Reference
Staphylococcus aureus	+	ATCC 6538
Streptococcus pneumoniae	+	ATCC 6505
Clostridium perfringens	+	ATCC 13124
Bacillus cereus	+	ATCC 14579
Pseudomonas aeruginosa	-	ATCC 10145
Escherichia coli	-	NTCC 11954
Salmonella typhi	-	ATCC 14028
Vibrio fischeri	-	ATCC 49387
Candida albicans		ATCC 10321

2.1.3. Animals

OF-1 strain Albino mice (*Mus musculus*), weighing 25 ± 2 g, were provided by the Pasteur Institute of Madagascar (IPM) breeding farm.

2.2. Methods

2.2.1. Extraction of the EOs

LEO and BEO were extracted by conventional hydrodistillation using a CLEVENGER apparatus type [6].

2.2.2. Physico-chemical characterization of LEO and BEO

The physical and chemical parameters to be determined and the references used are shown in Table 2

Table 2 Parameters to be determined and the standards used

Parameters	Standards used
Relative density	AFNOR, NF-T 75-111
Refraction index	AFNOR, NF-T 75-112
Rotation power	AFNOR, NF-T 75-13
Acid index	AFNOR, NF-T 75-103
Ester index	AFNOR, NF-T 75-104

2.2.3. Analysis of LEO and BEO

The chemical composition of essential oils was determined by gas chromatography coupled with mass spectrophotometry (CPG/MS). A chromatograph equipped with a Thermo Brand TRACE Network Mass Selective Detector and a DBWAX fused silica capillary column (30 m x 0.25 mm x 0.25 µm), was used. The peaks obtained were identified using AMDIS software, version 2.69 (Automated Mass Spectral Deconvolution and Identification System).

2.2.4. Assessment of antimicrobial activity

Antimicrobial susceptibility testing by the agar diffusion method

The sensitivity of microorganisms to the essential oil was determined by the agar diffusion method or aromatogram as described by [7]. Sterile paper disks (6 mm in diameter BioMérieux ®, REF 549916) were soaked with pure essential oil and placed on the surface of the inoculated Mueller-Hinton Agar (Scharlau®). The Petri dishes were incubated at 37°C for 24 h and the Inhibition Zones (IZ) were measured. The sensitivity to the essential oil was classified according

to the IZ diameter as: not sensitive (-) for IZ ≤ 8 mm; sensitive (+) for 9 ≥ IZ ≤ 14 mm; very sensitive (++) for 15 ≥ IZ ≤ 19 mm and extremely sensitive (+++) for IZ ≥ 20 mm [8, 9].

MIC and MBC determination

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined by microdilution method and the standards used to interpret results were those of [10]. The essential oil type of action is bactericidal when the ratio MBC/MIC is ≤4 or bacteriostatic when MBC/MIC is > 4 [11].

2.2.5. Toxicity determination

A volume of 0.3 ml of EO per 25 ± 2 g of body weight was administered to mice by oral route by means of an intubation cannula with a curved distal. Two batches of 5 male mice were used. The mice were observed for 24 h [12, 13].

2.3. Statistical analysis

The results were expressed as average values ± standard deviations from three separate determinations. One-way variance analysis (ANOVA) with XLSTAT 2014 software was used for statistical analysis. Statistical estimates were made at the 95% confidence interval.

3. Results and discussion

3.1. Extraction yields and physico-chemical parameters

The extraction yields and the physico-chemical parameters determined of LEO and BEO are presented in Table 3.

Table 3 Extraction yields and physico-chemical indexes of LEO and BEO

EO	yield%	Density	Rotation power	Acid index	Ester index	Refractive index
LEO	0.10	0.8631±0.0003	+15°76±0°17	1.9865±0.06	13.2865±3.25	1.4717±0.0002
BEO	0.10	0.8791±0.0004	+11°60±0°17	1.9573±0.03	18.7382±5.75	1.4799±0.0002

3.2. Chemical composition of LEO and BEO

The compounds identified in LEO and BEO with their respective percentages are presented in Figures 2 and 3 and Tables 4 and 5.

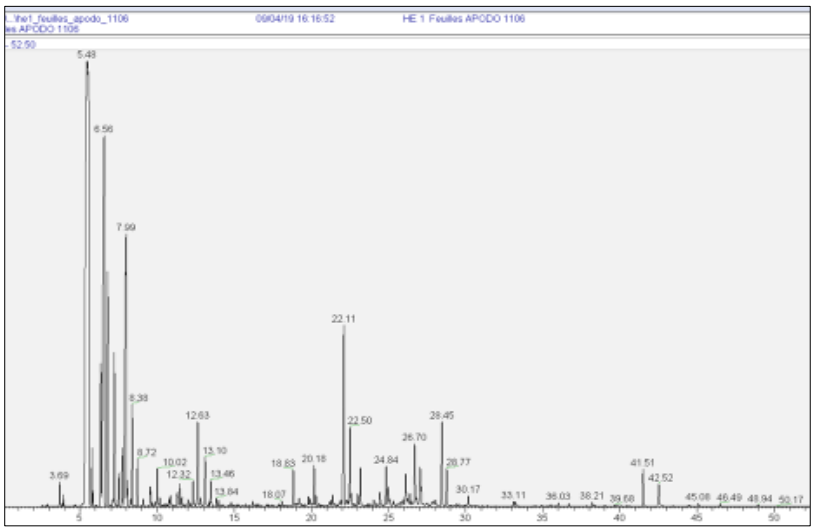

Figure 2 Chromatographic profile of LEO

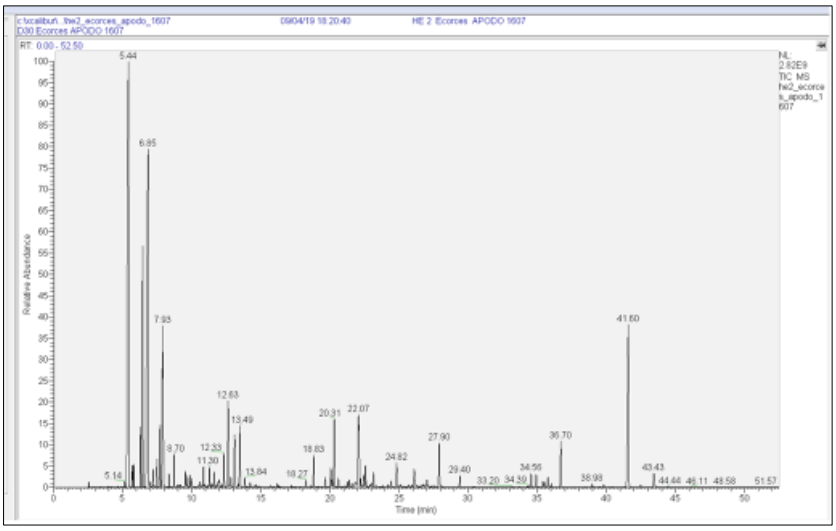

Figure 3 Chromatographic profile of BEO

Table 4 LEO components

Peak number	Retention time (min)	Component	Relative rate (%)
1	3.6887	Amylene	0.10
2	5.5728	α-pinene	27.5
3	5.8484	2.4(10)-thujadiene	0.11
4	6.3454	Sabinene	13.62
5	6.5554	β-pinene	12.01
6	6.7904	Myrcene	3.87
7	7.2358	α-phellandrene	8.56
8	7.4977	Terpinolene	0.33
9	7.7361	p-cymene	3.35
10	7.8926	Limonene	7.42
11	8.3733	Trans ocimene	1.15
12	8.7177	γ-terpinene	0.49
13	9.5407	Terpinene	0.14
14	9.5873	Trans linalool oxyde	0.39
15	9.6906	Dehydroparacymene	0.03
16	9.8687	Camphenone 6	0.04
17	10.0152	Linalol	0.45
18	10.1718	Nonanal	0.10
19	10.7808	Hydrate de sabinene trans	0.08
20	10.8814	α-campholene	0.09
21	11.3010	Cis-sabinol	0.16
22	11.4654	Cis-verbenol	0.24
23	11.993	Pinocarvone	0.06

24	12.3218	α-phellandrene 8-ol	0.4
25	12.6330	Terpinene 4-ol	1.25
26	13.1032	α-terpineol	0.48
27	13.4620	Verbenone (I)	0.44
28	13.9882	Ortho cymene	0.06
29	18.8324	Copaene	0.50
30	19.7942	α-gurjunene	0.10
31	19.9105	α-longipinene	0.07
32	20.2945	β-cedrene	0.13
33	22.1037	Copahu 6	3.64
34	22.4971	Elyxene	1.13
35	23.1739	δ-cadinene	0.49
36	24.4469	Nerolidol	0.19
37	24.8412	Spathulenol	0.54
38	24.9760	Caryophyllene oxyde	0.22
49	26.1089	Cubenol	5.07
40	26.6978	Basilic egypte essence	0.93
41	27.1159	Selina-6-en-4-ol	0.16
42	28.4473	β-caryophyllene	1.45
Total			97.54

Table 5 BEO components

Peak number	Retention time (min)	Component	Relative rate (%)
1	5.1421	α-thujene	0.12
2	5.3651	α-pinene	34.32
3	0.7317	Camphene	0.41
4	5.8048	2,4(10)-thujadiene	0.29
5	8.2890	Sabinene	14.53
6	6.4590	β-pinene	4.98
7	6.8413	Myrcene	15.1
8	7.1988	α-phellandrene	0.33
9	7.4923	Terpinolene	0.42
10	7.7213	p-cymene	1.79
11	7.8990	Limonene	2.25
12	8.3390	Trans ocimene	0.20
13	8.7019	γ-terpinene	0.52
14	0.5295	Terpinolene	0.45

15	9.6818	Dehydroparacymene	0.13
16	9.8615	Camphenone 6	0.19
17	0.9910	Linalol	0.18
18	10.8505	α-campholene	0.38
19	11.2985	L-pinocarveol	0.43
20	12.3264	Degracitral II	0.85
21	12.6333	Terpinene 4-ol	6.41
22	13.4881	Verbenone	1.27
23	13.8402	Trans carveol	0.16
24	18.8249	Copaene	0.70
25	19.6563	β-cadinene(-)	0.22
26	20.2490	α-cedrene	0.49
27	20.3082	β-cedrene	1.59
28	20.6097	Trans α-bergamotene	0.15
29	22.1080	γ-humulene	3.91
30	22.5816	β-cadinene(-)	0.42
31	23.1533	δ-cadinene	0.31
32	24.8160	Spathulenol	0.53
33	27.9016	α-bisabolol	1.62
34	29.3980	Nootkatone 1	0.25
35	34.5551	Dimyrcene 1	0.33
36	36.6976	Kaurene	1.18
37	41.4313	Scarlene	0.38
38	44.5675	β-pimaric acid	1.65
Total			99.44

3.3. Antimicrobial activity

The results of the evaluation of the antimicrobial activity of LEO and BEO are presented in Table 6.

Table 6 Antimicrobial activity (IZ in mm) of LEO, BEO (10 µl/disk)

Microorganisms	**LEO 8.62 mg/disk**	**BEO 8.78 mg/disk**	**Neomycin 30 µg/disk**	**Gentamycin 30 µg/disk**
Staphylococcus aureus	11	15.5	25	26
Sensitivity	+	++	+++	+++
Streptococcus pneumoniae	12.5	26	34	25
Sensitivity	+	+++	+++	+++
Clostridium perfringens	18.5	25.5	28	25
Sensitivity	++	+++	+++	+++
Bacillus cereus	12	13.5	29	24
Sensitivity	+	+	+++	+++
Pseudomonas aeruginosa	15	46	32	23
Sensitivity	++	+++	+++	+++
Escherichia coli	8	9.5	27	21
Sensitivity	-	+	+++	+++
Salmonella typhi	11	19.5	21	22
Sensitivity	+	++	+++	+++
Vibrio fischeri	13.5	20	20	18
Sensitivity	+	+++	+++	++
Candida albicans	15.5	25.5	41	33
Sensitivity	++	+++	+++	+++

LEO and BEO were active against the vast majority of microorganisms tested with IZs up to 46 mm for BEO on *Pseudomonas aeruginosa* with activity significantly higher than reference antibiotics.

The MIC, MBC and MBC/MIC of LEO and BEO are shown in Table 7.

Table 7 MIC, MBC and MBC/MIC of LEO and BEO on different microorganisms

Microorganisms		**LEO (µg/ml)**			**BEO (µg/ml)**		
		MIC	**MBC**	**MBC/MIC**	**MIC**	**MBC**	**MBC/MIC**
Gram negative	*Salmonella typhi*	107.6	215.3	2	13.7	13.7	1
	Vibrio fischeri	13.45	26.9	2	6.85	13.7	2
	Pseudomonas aeruginosa	215.3	215.3	1	219.3	219.3	1
	Escherichia coli	215.3	430.7	2	54.8	109.6	2
Gram positive	*Staphylococcus aureus*	107.6	215.3	2	13.7	27.4	2
	Streptococcus pneumoniae	107.6	107.6	1	219.3	219.3	1
	Clostridium perfringens	53.8	430.7	8	6.85	27.4	4
	Bacillus cereus	107.6	215.3	2	6.85	27.4	4
Yeast	*Candida albicans*	0.925	6.725	7	6.85	6.85	1

LEO and BEO showed MICs < 500 µg/ml on all microorganisms tested. For LEO, the MBC/MIC ratio was > 4 on *Clostridium perfringens* and *Candida albicans*, but < 4 for the other germs, while for BEO, the MBC/MIC ratio was ≤ 4 on all germs tested.

3.4. LEO and BEO toxicity

The acute toxicity indexes (LD_0, LD_{50} and LD_{100}) of LEO and BEO in mice by the oral route are presented in Table 8.

Table 8 Oral acute toxicity of LEO and BEO on mice

Dose (g/Kg body weight)	LEO	BEO
LD_0	1.288	2.630
LD_{50}	2.478	2.343
LD_{100}	5.168	5.264

By oral route, at the doses tested corresponding to LD_0, LD_{50} and LD_{100}, the symptoms are the same, but their intensity and duration as well as the mortality rate were dose dependent. They included itchy muzzle, decreased motor activity, body tremor, loss of appetite, sweating, piloerection, enophthalmos, drowsiness and dyspnea. At the LD_0 dose, the symptoms gradually decreased and disappeared after 4 h and the animals have fully recovered after 24 h, whereas at the LD_{100} dose, the symptoms intensified and all animals died after 24 h.

4. Discussion

The *A. pauciflora* EOs were obtained with yields of 0.1% which are much lower than those of *Helichrysum ibityense* (1.9%) [14] and *Senecio longiscapus* (3%) [9], two Malagasy Asteraceae. According to [15], the yields of essential oils are extremely variable depending on the plants considered, but they are generally very low, below 1%.

With similar density values (0.8631 and 0.8791 respectively) well below 1 (density of water), LEO and BEO were light oils. They are light yellow in colour and have a strong odour. They are dextrorotatory and their acid indexes were less than 2 which is an indicator of a good conservation [16]. Their ester indexes (< 20) were higher than that of *Senecio longiscapus* leaves (12.49) [9], but significantly lower than that of *Helichrysum ibityense* leaves [14]. In several EOs considered to be of good quality, the ester index values are much higher: *Kaempferia galanga* rhizome EO (189.65) [17]; *Cananga odorata* flower EO, (350.6) [16]. The refractive index values of LEO and BEO were similar (1.4717 and 1.4799 respectively). According to [18], the low refractive index of essential oils (1.4710 to 1.4880) indicate their low refraction of light, which could favour their use in cosmetic products.

The chemical compositions of LEO and BEO differed in the total number of components, the levels of the main common components and the existence of non-common compounds. Forty-two (42) compounds, representing 97.54% of the overall composition, were found in LEO versus 38 compounds representing 99.44% in BEO. More than 50% of the major components with levels above 10% are all monoterpenes. In LEO, the major components were α-pinene (27.5%), sabinene (13.62%) and β-pinene (12%) and in BEO, α-pinene (34.32%), myrcene (15.1%), sabinene (14.53%). Regarding the differences in the levels of some common products, limonene was present at 7.42% in LEO versus 2.25% in BEO, while terpinene 4-ol was at 1.25% in LEO versus 6.41% in BEO. Among the non-common components for the 2 EOs, cubenol, 5.07% in LEO was absent in BEO and conversely, γ-humulene, 3.91% in BEO was absent in LEO.

All microorganisms tested by the agar diffusion method were susceptible to both LEO and BEO, but BEO was significantly more effective. In some cases, BEO (heterogeneous mixture) was as effective as neomycin (pure product), or even more effective: for example, against *Clostridium perfringens*, IZ = 25.5 mm for BEO versus IZ = 25 mm for neomycin; against *Pseudomonas aeruginosa*, IZ = 46 mm for BEO versus IZ = 23 mm for neomycin. By the microdilution method, LEO and BEO were active against all germs tested with different intensities depending on the EO and the germ tested. According to [10], LEO activities were excellent (MIC<100 µg/ml) against 33.3% of the germs tested and moderate (100<MIC<500 µg/ ml) against 66.7%. For BEO, it was the opposite, 66.7% excellent and 33.3% moderate. With standards used by other authors, plant extracts with MIC values higher than 500 µg/ ml [19] and even much higher than 1000 µg/ml [20, 21, 22] were classified as having strong antimicrobial activity.

LEO action was bacteriostatic against *Clostridium perfringens* and *Candida albicans* but bactericidal against the other germs tested whereas that of BEO was bactericidal for all germs.

The major components in the 2 EOs are all well known for their antimicrobial activities: α-pinene [23, 24]; sabinene [25, 9]; β-pinene [23, 14] and myrcene [25, 26]. However, some studies have shown that the use of the whole essential oil provides an effect which is greater than of major components used together [27, 28]. This suggests that minor components are essential for activity and may have a synergistic effect [28]. Therefore, as example, limonene [29] and γ-terpinene [30] which were among the minor components of LEO and BEO, might contribute to the antimicrobial activity of these EOs.

LEO and BEO have similar oral LD_{50} of 2.478 and 2.343 g/Kg body weight respectively. These values were relatively high [31], which meant that the 2 EOs were weakly toxic. However, other toxicological studies such as subchronic and chronic toxicity, impacts on major physiological functions (cardiac, renal and hepatic), etc. will still be needed to better determine the acceptable conditions for the possible use of these EOs.

It is known that the extraction yield of an EO, the composition and level of its constituents can vary significantly depending on several parameters including the physiological stage of the plant, which could significantly modify its biological properties. Therefore, various works are in progress in our laboratory to deepen the knowledge on the essential oils of *Apodocephala pauciflora*: analysis of the EOs of plant parts collected at different times to determine the most appropriate time for harvesting; search of other known biological properties of components that are part of the main components of LEO and BEO such as antioxidant, anti-inflammatory activities. Work on antifungal properties for food protection has already shown promising results.

The extension of the present work to other known species of the genus *Apodocephala* is already planned.

5. Conclusion

This is the first report on the constituents and pharmacological activities of *Apodocephala pauciflora* EOs. Their chemical composition and physicochemical characteristics are determined. They contain constituents known for their interesting biological properties. The first data on their antimicrobial activity are promising and their low toxicity is interesting for their possible uses. *Apodocephala pauciflora* constitutes a potential source of interesting and accessible therapeutic molecules. These first results contribute to the knowledge of the endemic genus *Apodocephala* and the aromatic plants of the Mandraka forest.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interests.

Statement of ethical approval

All the tests on animals were approved and in line with the standard established by Ethics Committee of Pasteur Institute of Madagascar.

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