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Antimicrobial and Cytotoxicity Activity of *Clausena anisata*, *Acokanthera shemperii* and *Olea europaea* Growing in Tanzania

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Authors' contributions

All authors worked together to achieve this work. All authors have cordially supported the work and preparation of the manuscript. Author WEM designed and supervised the study and prepared the first draft of the manuscript. Authors MC and LJC advised and guided the final draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate antimicrobial and cytotoxicity activities of *Clausena anisata*, *Acokanthera shemperii* and *Olea europaea* against seven Gram negative bacteria and fungal species.
Study Design: Bioassay of antimicrobial assay was done using 96-well micro-dilution method.
Place and Duration of Study: School of Life Science and Bioengineering, Nelson Mandela African Institution of Science and Technology, Arusha, Tanzania, from April 2014 to June 2014.
Methodology: 96-well micro dilution method was used in antimicrobial assay. Extracts were loaded in the wells of the first row, followed by serial dilution and 50 µl of the bacterial suspensions (0.5 MacFarland standard turbidity) were added in each well. The first concentration which showed no bacterial growth was considered as minimum inhibition concentration. Method developed by Meyer et al 1982 was adopted in cytotoxicity activities.
Results: All extracts indicated antibacterial activity on at least three to five of the tested seven bacteria and two fungi species with MIC value ranging 0.7812 - 12.5 mg/mL. The highest activity

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was demonstrated by *Olea europaea* leaf methanolic, *Acokanthera shemperii* stem bark and *Clausena anisata* twigs ethyl acetate extracts with MIC value of 0.7812 mg/mL against *Pseudomonas aeruginosa* while the same MIC value was exhibited by *Olea europaea* stem bark methanol against *Proteus mirabilis.* However the *Olea europaea* root methanolic extract inhibited the growth of *Pseudomonas aeruginosa* and *Salmonella kisarawe* with MIC value of 0.7812 mg/mL. *Olea europaea* leaf methanolic and stem bark methanolic which demonstrated high antimicrobial activity were non toxic against brine shrimp larvae with LC_{50} value of 369.8272 and 226.1566 µg/mL, while *Clausena anisata* twigs ethyl acetate, *Acokanthera shemperii* stem bark ethyl acetate and *Olea europaea* root methanolic extracts were toxic with LC_{50} value of 6.21276, 67.4179 and 92.3089 µg/mL respectively.

Conclusion: This study has unveiled antimicrobial and cytotoxicity properties of *Clausena anisata*, *Acokanthera shemperii* and *Olea europaea.*

Keywords: Antimicrobial; Clausena anisata; Acokanthera shemperii; Olea europaea.

1. INTRODUCTION

Antibiotics and antimicrobial agents have been used to treat infectious diseases for the last 70 years. Since 1940's antibiotics were reported to reduce the burden that the infectious diseases pose on human health [1]. However, the long term use of these drugs have given a chance to microorganism to adopt and make the drugs less effective [2]. It is known that, bacterial and some fungal species have ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents [3]. The emergence of multi-drug resistant pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Haemophilus* and *Candida albicans* has been a major challenge in the management of diseases caused by these pathogens [4,5]. For instance, in Tanzania, blood stream infection caused by resistant Gram negative bacteria and *C. albicans* is accounted to be a main source of mortality to children, with 34.9% mortality rate [6]. Therefore, the evidence of the rapid global spread of resistant clinical isolates necessitated the search for new class of drugs with unique mode of action [7,8]. However, there is widespread of resistance to new introduced antimicrobial agent which specify the short life expectancy for the new family of the introduced drugs [9]. Action must be taken to address and safeguard health and life in general for the poor communities which are the most vulnerable. The validation of ethnomedical information so as to develop herbal products from medicinal plants is of great interest to researchers.

Medicinal plants have been used as a primary health care by about 80% of individuals in the developing countries for many years [10]. For instance, communities in Tanzania use *Clausena anisata* leaves for treatment of oral candidiasis [11]. This plant is also used by communities in West Africa for treatment of boils, ringworm, oral thrush and eczema [11]. Likewise the majority of sexually transmitted diseases in Tanzania and Kenya are treated by infusion of the pounded root of *Acokanthera schemperii* [12]. Moreover *Olea europaea* is extensively used to treat diarrhea, respiratory and urinary tract infections, stomach and intestinal diseases, and as mouth cleanser by different communities around the world [13]. In East-Africa infusion from *Olea europaea* bark is taken for tapeworm infestation [13]. Despite the contribution of medicinal plants for management of diseases, only limited number of medicinal plants have been scientifically validated [14]. Thus, this study reports the antimicrobial and cytotoxicity activity of *Acokanthera shemperii*, *Olea europaea* and *Clausena anisata* growing in Tanzania.

2. MATERIALS AND METHODS

2.1 Chemicals and Organisms Tested

Chloroform, ethyl acetate, methanol and dimethyl sulfoxide (DMSO) was purchased from Avantor performance materials India. Fluconazole was acquired from Lincoin Pharmaceuticals LTD, India, ciprofloxacin were bought from Micro Lab LTD, India and cyclophosphamide was bought from Khandelwa Laboratories Pvt Ltd (Mumbai), iodonitrotetrazolium chloride (INT) was purchased from SIGMA (Sigma Aldrich, St Louis, USA). Nutrient (agar and broth), sabouraud dextrose (agar and broth) were purchased from Hi Media Laboratories Pvt Ltd (Mumbai-India). Sea salt was prepared locally by evaporating water collected from the Indian Ocean, along the Dar es salaam Coast. *Klebsiella oxytoca* (clinical isolate), *Klebsiella pneumoniae* (ATCC700603), *Proteus mirabilis* (NCTC 1075), *Salmonella typhi*

(NCTC 8385), *Salmonella kisarawe* (clinical isolate), *Pseudomonas aeruginosa* (ATCC 29953), *Escherichia coli* (ATCC 25922), *Candidas albicans* (ATCC 90028) and *Cryptococcus neoformans* (clinical isolate) were obtained from the Department of Microbiology, Muhimbili University of Health and Allied Sciences (MUHAS).

2.2 Sample Collection

Fruits, leaves, stem bark, twigs and roots of *C. anisata*, A. *schimperi* and *O. europaea* were collected from Monduli in Arusha region, Tanzania on 26th January 2015. Plant species were identified by a Botanist from Tropical Pesticides Research Institute (TPRI) Arusha, and the voucher specimens (CA300, AS201 and OE102) were kept at Nelson Mandela African Institute of Science and Technology, Arusha.

2.3 Extraction Process

Plant materials were under shade dried and pulverized into powder form for extraction. 800 g of the pulverized plant parts (fruits, leaves, twigs, stem bark and roots) were sequentially macerated using chloroform, ethyl acetate and methanol for 48 hrs twice for each solvent. The respective extracts were filtered through Whatman filter paper number 1 in a glass column. The crude extracts were obtained after subjecting the macerated plant materials in a rotary evaporator and the extracts were stored in refrigerator at -20℃ until testing time.

2.4 Testing for Antimicrobial Activity

Extracts were tested against representative Gram negative bacteria; *S. typhi* (NCTC 8385), *P. aeruginosa* (ATCC 29953), *S. kisarawe* (clinical isolate), *E. coli* (ATCC 25922), *K. oxytoca* (clinical isolate), *K. pneumoniae* (ATCC700603), *P. mirabilis* (NCTC 1075), *C. albicans* (ATCC 90028) and *C. neoformans* (clinical isolate). Selection of the microorganism was based on their availability during the experiment. Minimum inhibitory concentrations (MICs) were obtained using microdilution method. Stock solution was prepared by dissolving 100 mg of extract in 1 mL of DMSO (100 mg/mL). Each of the 96 well microtitre plates were first preloaded with 50 µl of broth media, followed up by 50 µl of extracts in first well of each row which make up 100 µl total volume in the first wells. Subsequently 50 µl were transferred from the first rows of each category to the second rows and the process was repeated down the columns on which the last wells at the bottom 50 µl were discarded. Gentamicin (100 µg/mL) was used as standard drug (positive control); DMSO as negative control and the row contains only broth as growth control. Thereafter 50 µl of the bacterial suspensions (0.5 MacFarland standard turbidity) were added in each well and incubated at 37 ℃ for 24 h. 40 µl of 0.02% p-iodonitrotetrazolium (INT) chlo-ride solution was added to each well followed by incubating for 1 h at 37℃. Bacteria growth was indicated by change in color of INT (pink color). Absence of bacterial was indicated with no change in dye's color. The first concentration which showed no bacterial growth was considered as Minimum inhibition concentration (MIC).

2.5 Brine Shrimps Lethality Test

Brine shrimps lethality test was conducted as described by Meyer et al. [15]. Stock solution was prepared by dissolving 40 mg of extracts in 1 mL DMSO (40 mg/mL). Different concentration levels (240, 120, 80, 40, 24 and 8 µg/mL) was prepared by drawing different volume from stock solution and was added in a 10 mL universal bottle containing 10 brine shrimps larvae. Volume was then adjusted to 5 mL with artificial sea water prepared by dissolving 3.8 g of sea salt in 1 L of distilled water and incubated under light for 24 hrs. Each concentration was tested in duplicate for statistical significance. Negative controls contain brine shrimp, DMSO and artificial sea water and cyclophosphamide was used as a positive control. Dead larvae were identified and the mean results of the percentage mortality were plotted against the logarithms of concentrations using the Fig P computer program. LC_{16}, LC_{50}, LC_{84} and the 95% CI values were calculated from the Regression equation obtained from the graph.

3. RESULTS

3.1 Antimicrobial Activity

All extracts from the selected plants demonstrated antimicrobial activity on at least three to five of the tested bacteria and fungi species with minimum inhibition concentration (MIC) value ranging 0.7812-12.5 mg/mL (Table 1). The highest activity of MIC value 0.7812 mg/mL was demonstrated by

Olea europaea root methanolic extract, *Acokanthera shemperii* stem bark ethyl acetate and *Clausena anisata* twigs ethyl acetate extracts against *Pseudomonas aeruginosa.* The same MIC value was also exhibited by *O. europaea* stem bark methanolic extract against *Proteus mirabilis*. A number of extracts demonstrated antimicrobial activity with MIC value of 1.5625 mg/mL against the tested pathogens. For instance *C. anisata* leaf chloroform, ethyl acetate and methanolic extracts exhibited MIC value of 1.5625 mg/mL against *Salmonella typhi*. Similarly *C. anisata* twigs ethyl acetate indicated the same MIC value of 1.5625 mg/mL against *S. typhi*, *Escherichia coli*, *Salmonella kisarawe* and *Klebsiella oxytoca.* Likewise *C. anisata* stem bark chloroform and *A. shemperii* leaf ethyl acetate inhibited the growth of *S. typhi*, *Klebsiella oxytoca* and *P. aeruginosa* with MIC values of 1.5625 mg/mL. Another extracts that demonstrated the MIC value of 1.5625 mg/mL are *A. shemperii* stem bark chloroform and *A. shemperii* root chloroform against *P. aeruginosa.* Furthermore the MIC value of 1.5625 mg/mL was also showed by *O. europaea* leaf methanolic against *S. typhi*, *E. coli* and *Klebsiella pnemoniae*. Similarly *O. europaea* roots methanolic exhibited the same MIC value of 1.5625 mg/mL against *S. typhi*, *K. oxytoca* and *K. pnemoniae*. Minimum inhibitory concentrations ranging from 3.125-12.5 mg/mL were indicated by the remaining extracts. Despite the fact that Rios and Recio (2005), recommended the maximum concentration of interest should be less than 1 mg/mL, however the extracts with low antimicrobial activity should also be reported as it can be incorporated with other extracts to improve it biological importance.

3.2 Brine Shrimp Lethality Tests

Extracts were evaluated for lethality activity against the brine shrimp larvae and results are summarized in Table 2. According to Meyer et al [15], extracts that demonstrates LC_{50} value greater than 100 µg/mL was considered as non-toxic and LC_{50} value less than 100 µg/mL as toxic. *Acokanthera shemperii* root ethyl acetate, *C. anisata* leaf chloroform, *C. anisata* twigs ethyl acetate and *A. shemperii* root chloroform were more toxic with LC_{50} less than 20 µg/mL.

4. DISCUSSION

Healing properties of medicinal plants that are used to manage infectious diseases has been proven by several studies conducted to evaluate antimicrobial activities of medicinal plants [16,17]. Medicinal plants are known to cure infectious disease such as urinary tract infections, gastrointestinal disorders, respiratory diseases and cutaneous infections [18,19]. The antimicrobial activity of plants materials against bacteria and fungi strains is due to their chemical composition [20]. Since secondary metabolites are produced in response to fungal and bacterial challenges that plant is facing.

Secondary metabolites produced by a medicinal plant in one region may be similar or different from the same species in another region. For instance *Clausena anisata*, *Acokanthera shemperii* and *Olea europaea* have been exploited in different parts of Africa for management of infectious diseases [21-23]. These uses prompted scientists to validate the antimicrobial properties of *C. anisata*, *A. shemperii* and *O. europea*. For instance the study conducted on *O. europaea* leaf acetone extract inhibited the growth of *Salmonella enteritidis*, *Bacillus cereus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterococcus faecalis*, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* [24]. Likewise the study conducted in Ethiopia on the *A. schimperi* methanolic and water extracts showed the growth inhibition of *S. aureus*, *S. pyogens*, *E. coli*, *P. aeruginosa* and *P. vulgaris* [25]. It is however interested to observe that the current study revealed that chloroform, ethyl acetate and methanolic extracts of *C. anisata*, *A. shemperii* and *O. europaea* growing in Tanzania had antimicrobial activity with MIC value ranging between 0.7812-25 mg/mL against microbes tested in this study. Furthermore the investigations conducted on the leaf essential oil from *C. anisata* growing in India demonstrated the inhibition effects on *S. typhi* and *P. aeruginosa* with the minimum inhibitory concentration (MIC) values of 62.5 and 125 µg/mL, respectively [26]. Results of antimicrobial activity might differ due to the specifics of collection, solvent and the method used for extraction [27]. Furthermore, Rios et al. [27] proposed that the antimicrobial activity is of interest when the concentration is below 100 µg/mL, but weak compounds should be used with other compounds to improve their activities. Thus based on that study, extracts that show antimicrobial activity in this study can be used with other extracts or with other compounds when isolated to improve its activity.

Table 1. Antimicrobial activity *Clausena anisata*, *Acokanthera shemperii* and *Olea europaea*

Extracts	Minimum inhibition concentration MIC (mg/mL)								
	S. typhi	***P. mirabilis***	***E. coli***	***S. kisarawe***	***K. oxytoca***	***K. pnemoniae***	***P. aeruginosa***	***C. albicans***	***C. neoformans***
CALC	1.5625	3.125	6.25	3.125	3.125	3.125	3.125	3.125	1.5625
CALE	1.5625	3.125	6.25	3.125	6.25	3.125	3.125	6.25	1.5625
CALM	1.5625	3.125	12.5	6.25	6.25	3.125	3.125	6.25	3.125
CATC	6.25	3.125	12.5	3.125	6.25	3.125	3.125	25	12.5
CATE	1.5625	3.125	1.563	1.563	1.5625	6.25	0.7812	12.5	6.25
CATM	3.125	3.125	3.125	3.125	3.125	6.25	1.5625	12.5	6.25
CASBC	1.5625	6.25	3.125	6.25	1.5625	6.25	1.5625	12.5	6.25
CASBM	3.125	6.25	3.125	6.25	6.25	6.25	6.25	12.5	12.5
CAFC	3.125	3.125	6.25	6.25	6.25	6.25	3.125	12.5	12.5
CAFE	3.125	3.125	6.25	3.125	3.12	6.25	6.25	12.5	3.125
CAFM	3.125	3.125	3.12	6.25	3.12	12.5	3.12	12.5	1.5625
ASLC	12.5	12.5	12.5	12.5	6.25	12.5	6.25	12.5	3.125
ASLE	1.5625	12.5	12.5	6.25	1.5625	12.5	1.5625	12.5	3.125
ASLM	12.5	12.5	12.5	3.125	12.5	3.125	3.125	12.5	12.5
ASSBC	6.25	12.5	12.5	6.25	6.25	6.25	1.5625	25	25
ASSBE	6.25	6.25	6.25	6.25	6.25	6.25	0.78125	12.5	6.25
ASSBM	12.5	12.5	12.5	6.25	6.25	12.5	3.125	12.5	12.5
ASRC	12.5	12.5	12.5	12.5	12.5	12.5	1.5625	12.5	6.25
ASRE	12.5	6.25	12.5	12.5	6.25	6.25	3.125	12.5	3.125
ASRM	6.25	12.5	6.25	6.25	3.125	3.125	6.25	25	12.5
OELC	12.5	6.25	12.5	12.5	25	12.5	12.5	12.5	6.25
OELE	3.125	6.25	3.125	6.25	3.125	6.25	3.125	12.5	3.125
OELM	1.5625	3.125	1.562	6.25	0.7812	1.5625	6.25	12.5	6.25
OESBC	6.25	6.25	12.5	12.5	25	12.5	12.5	6.25	25
OESBE	3.125	12.5	3.125	6.25	6.25	3.125	6.25	25	12.5

Extracts	Minimum inhibition concentration MIC (mg/mL)								
	S. typhi	***P. mirabilis***	***E. coli***	***S. kisarawe***	***K. oxytoca***	***K. pnemoniae***	***P. aeruginosa***	***C. albicans***	***C. neoformans***
OESBM	3.125	0.7812	3.125	1.5625	3.125	3.125	6.25	25	3.125
OERC	12.5	12.5	12.5	6.25	12.5	12.5	12.5	25	25
OERE	6.25	12.5	12.5	6.25	6.25	25	3.125	25	6.25
OERM	1.5625	6.25	3.125	0.7812	1.5625	1.5625	0.7812	25	6.25
OETC	6.25	3.125	6.25	6.25	12.5	6.25	12.5	25	6.25
OETE	3.125	6.25	6.25	6.25	6.25	25	6.25	25	6.25
OETM	3.125	6.25	3.12	12.5	6.25	6.12	12.5	12.5	25
Cipro	0.391	0.7812	0.391	0.7812	0.7812	0.391	0.391	NA	NA
Fluco	NA	NA	NA	NA	NA	NA	NA	1.5625	0.7812

Key: CALC- C. anisata leaf chloroform, CALE- C. anisata leaf ethyl acetate, CALM- C. anisata leaf methanolic, CAFC- C. anisata fruits chloroform, CAFE- C. anisata fruits ethyl acetate, CAFM- C. anisata fruits methanolic, CATC- C. anisata twigs chloroform, CATE- C. anisata twigs ethyl acetate , CATM- C. anisata twigs methanolic, CASBC- C. anisata stem bark chloroform, CASBE- C. anisata stem bark ethyl acetate, CASBM- C. anisata stem bark methanolic, ASLC- A. shemperii leaf chloroform , ASLE- A.shemperii leaf ethyl acetate, ASLM- A. shemperii leaf methanolic, ASSBC- A. shemperii stem bark chloroform, ASSBE- A. shemperii stem bark ethyl acetate, ASSBM- A. shemperii stem bark methanolic, ASRC- A. shemperii root chloroform, ASRE- A. shemperii root ethyl acetate, ASRM- A. shemperii root methanolic, OELC – O. europaea leaf chloroform, OELE- O. europaea leaf ethyl acetate, OELM- O. europaea leaf Methanol, OESBC- O. europaea stem bark chloroform, OESBE- O. europaea stem bark ethyl acetate, OESBM- O.europaea stem bark methanol, OETC- O. europaea stem bark twigs chloroform, OETE- O. europaea twigs ethyl acetate, OETM- O. europaea twigs ethyl acetate, OERC- O. europaea roots chloroform, OERE- O. europaea roots ethyl acetate, OERM- O. europaea roots methanol, Cipro- ciprofloxacin, Fluco- Fluconazole and NA-not applicable

Table 2. Brine shrimp lethality tests of *Clausena anisata*, *Acokanthera shemperii* and *Olea europaea*

Extract	LC50 (µg/ml)	95% (confidence interval)	R^2	Regression equation
CALC	3.5761	1.9119-6.68865	0.902	y = 34.634logx + 30.833
CALE	36.6689	26.7263-50.30973	0.9763	y = 62.591logx - 47.911
CALM	127.7264	79.2052-205.9715	0.9851	y = 41.428logx - 37.259
CATC	115.0821	94.8258-139.6653	0.8885	y = 125.23logx - 208.1
CATE	6.1276	3.6043-10.4172	0.9857	y = 45.688logx + 14.03
CATM	28.446	21.6340-37.4025	0.9699	y = 79.221logx - 65.189
CASBC	99.0329	69.1918-141.7438	0.9738	y = 55.209logx - 60.185
CASBM	122.419	71.3323-210.0927	0.9657	y = 36.653logx - 26.526
CAFC	20.1162	13.8802-29.1540	0.9488	y = 53.35logx - 19.479
CAFE	70.045	50.8541-96.4780	0.9363	y = 61.829logx - 64.098
CAFM	1226.557	522.623-2878.631	0.9149	y = 23.205logx - 21.673
ASLC	37.8853	23.113-62.0996	0.9211	y = 40.059logx - 13.232
ASLE	808.289	370.162-1764.989	0.9418	y = 25.348logx - 23.701
ASLM	56.003	42.043-74.5982	0.9162	y = 69.046logx - 70.707
ASSBC	125.8478	78.668-201.3215	0.9741	y = 42.135logx - 38.477
ASSBE	67.4179	29.425-154.4678	0.9021	y = 23.878logx + 6.3325
ASSBM	222.0638	122.924-401.1586	0.8888	y = 33.474logx - 28.546
ASRC	16.597	12.239-22.507	0.8727	y = 79.6logx - 47.115
ASRE	14.3511	9.976-20.6448	0.9039	y = 59.636logx - 18.992
ASRM	71.8351	53.9871-95.5835	0.904	y = 69.308logx - 78.659
OELC	247.7954	85.7587- 715.991	0.9914	y = 18.657logx + 5.3334
OELE	50.3363	29.70- 85.3109	0.8906	y = 37.532logx - 13.875
OELM	369.8272	249.0529-549.169	0.947	y = 70.81logx - 131.84
OESBC	1627.450	642.219-4124.125	0.9682	y = 21.29logx - 18.373
OESBE	1164.063	468.1878-3359.85	0.8774	y = 21.735logx - 16.639
OESBM	226.1566	141.643-361.097	0.9357	y = 42.307logx - 49.608
OERC	11266.861	3206.30-39591.4	0.9274	y = 15.752logx - 13.824
OERE	67.4264	36.1951-125.6060	0.9462	y = 31.821logx - 8.1952
OERM	92.3089	52.0523-163.699	0.9109	y = 34.555logx - 17.909
OETC	52.9775	21.1128-132.934	0.9616	y = 21.518logx + 12.901
OETE	599.565	299.126-1201.76	0.8782	y = 28.47logx - 29.085
OETM	285,991.20	38126.4-2145260	0.9019	y = 9.8242logx - 3.6043
Cyclopho sphamide	16.37	12.01-22.31	0.995	y=69.97logx-34.936

In Tanzania leaves of *C. anisata* are used for management of skin fungal infections and oral candidiasis. Since the extracts from our study indicated activity against *C. albicans* and *C. neoformans*, extracts could be useful for treatment of fungal infections. Brine shrimp results indicated that some of the extracts that demonstrated antimicrobial activity are nontoxic while others are toxic. According to Meyer et al [15], extracts with LC_{50} value less than 20 µg/mL are considered as potential anticancer agents. In this study *C. anisata* leaf chloroform, *C. anisata* twigs ethyl acetate, *A. shemperii* root chloroform and *A. shemperii* root ethyl acetate extracts which demonstrated LC_{50} value of 3.5761, 6.1276, 16.597 and 14.3511 µg/mL respectively are potential anticancer agents. It is of high interest that our study displayed two advantages for therapeutic industry, as compounds from these extracts are potential antimicrobial and anticancer drug leads. Thus the use of *C. anisata*, *O. europaea* and *A. shemperii* is therefore validated in this study.

5. CONCLUSION

This study revealed antimicrobial and cytotoxicity activity of C*lausena anisata*, *Olea europaea* and *Acokanthera shemperii*.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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