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Chemical composition, antibacterial and antimycoplasma activities of four *Eugenia* species growing in Brazil

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The aim of this study was to evaluate the chemical composition and antimicrobial activity of four unexplored *Eugenia* species: *Eugenia brevistyla, Eugenia handroana, Eugenia catharinae* and *Eugenia stigmatosa.* Eight extracts and eighteen fractions were screened for their antibacterial activity against some selected bacteria including *Mycoplasma*. Phytochemical screening revealed that the plants were rich in terpenes and phenolic compounds. Antimicrobial evaluation revealed that *E. handroana* (FAEF-EH) and *E. brevistyla* (FAEF-EB) had the highest activity against *Staphylococcus aureus* with minimum inhibitory concentration (MIC) of 15.62 µg/ml *Mycoplasma* while FAEF-EH also presented the best activity with MIC of 62.5 µg/ml against *Mycoplasma pneumonia* M129. Some isolated compounds, betulinic acid and glutinol, also exhibited antibacterial property against some bacteria used in the study. All the four species studied presented promising antibacterial activity while the active principles are yet to be elucidated.

Key words: *Eugenia handroana, Eugenia brevistyla, Eugenia catharinae, Eugenia stigmatosa,* antibacterial activity, antimycoplasma activity.

INTRODUCTION

The plants of the *Eugenia* genus (Myrtaceae) are widely distributed in tropical and subtropical regions (Fischer et al., 2005; Zaki et al., 2013). They are known for their tasty fruits, and include some Brazilian plants as *Eugenia uniflora* ("Pitanga"), *Eugenia involucrata* ("Cereja-do-mato"), *Eugenia jambolona* ("jambolão") and *Eugenia edulis* ("jaboticaba"). This genus comprises a large group of medicinal plants with therapeutic applications of their different medicinal properties, such as anti-inflammatory, hypoglycemic, diuretic, analgesic, antidiarrheal, anti-rheumatic, antibacterial, protection against stomach disorders, etc (Saha et al., 2002; Auricchio and Bacchi, 2003; Bag et al., 2012; Victoria et al., 2012; Famuyiwa and Adebajo, 2012; Garmus et al., 2014).

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Infections caused by numerous microorganisms are a constant public health problem, and bacterial infections are responsible for several diseases such as pneumonia, meningitis, and endocarditis (Souza et al., 2004). Infections caused by resistant microorganisms and those caused by "commensal" microorganisms, are increasing every year (Muraina et al., 2009). Mycoplasmas are bacteria that belong to the class *Mycoplasma*, and cause respiratory and urogenital diseases in human beings (Muraina et al., 2009). They are the smallest microorganisms that are capable of self-replication, lacking a cell wall and presenting variable susceptibility to antibiotics (Murray, 2007).

Considering the chemotaxonomy and the important activities already described for the *Eugenia* genus, the reported increase in antibiotic resistance, as well as the increase in infections caused by *Mycoplasma*, four Brazilian plants of the *Eugenia* species *E. brevistyla, E. handroana, E. catharinae* and *E. stigmatosa* have been evaluated for their chemical compositions and antimicrobial activity against selected bacteria including *Mycoplasma.*

MATERIALS AND METHODS

Plant material

The place and season (year) of collection of each species are shown in Table 1.

Phytochemical analyses

Extraction procedures

Leaves (760 g) and stems (495 g) of *E. brevistyla;* leaves (2056 g) and stems (980 g) of *E. handroana*; leaves (785 g) and stems (104 g) of *E. catharinae* and leaves (295 g) and stems (150 g) of *E. stigmatosa* were extracted separately by macerating in methanol for 7 days at room temperature. The solutions were then filtered and concentrated in a rotary evaporator under reduced pressure (50°C), furnishing the respective methanolic extracts. All the obtained extracts were successively partitioned with solvents of different polarities (dichloromethane or chloroform and ethyl acetate) to obtain the respective fractions (FDCM or $FCHCl_3$ and FAE). All the yields are shown in Table 2.

Isolation of the chemical constituents

In order to isolate the major compounds, all the fractions were separately subjected to open silica gel column chromatography (CC) eluted with hexane: ethyl acetate gradient. Thin layer chromatography, used to monitor purity, was carried out on a pre-coated Merck Kieselgel 60 F_{254} plate (0.25 mm) eluted with hexane: ethyl acetate gradient and the spots were compared with the standards. The compounds were identified by conventional spectral data (NMR, IR, MS) and compared with standard samples and the literature. In some cases, the compounds were subjected to gas mass spectrometry (GC/MS) to confirm their identities. The isolated compounds and the yields are shown in Table 3. The molecular structures are shown in Figure 1. The RMN spectral dates were:

β amyrin: NMR ^{1}H (300 MHz, $CDCl_3$, TMS): (ppm) 0.79 (s, 3H, H23), 0.83 (s, 6H, H30), 0.87 (s,3H,29), 0.94 (s, 6H, 24), 0.97; 0.99; 1.13 (s, 3x 3H; H26, H28, H27); 1.77 (dd, J= 4.3; 13.5 Hβ), 1.88 (dd, J= 4.0; 14.0 Hβ), 1.93 (dd, J= 4.0; 13.7 Hβ), 3.21 (dd, J= 4.3; 10.9). NMR ^{13}C (75 MHz, $CDCl_3$, TMS): (ppm) 15.5 (C25), 15.5 (C24), 16.8 (C26), 18.3 (C6), 23.5 (C11); 23.6 (C30), 25.9 (C27), 26.1 (C16), 26.9 (C15), 27.2 (C2), 28.1 (C23); 28.4 (C28), 31.0 (C20), 32.4 (7), 32.6 (C17), 33.3 (C29), 34.7 (C21), 36.9 (C10), 37.1 (C22), 38.5 (C4), 38.7 (C1), 39.8 (C8), 41.7 (C14), 46.8 (C19), 47.2 (C18), 47.6 (C9), 55.1 (C5), 79.0 (C3), 121.3 (C12), 145.2 (C13). The mixture of α-β amyrin was submitted to GC-MS, indicating the presence of 90% of β-amyrin and 10% of α-amyrin.

Nerolidol: Nerolidol, being an oil was identified by liquid chromatography coupled to mass spectrometry. The fragmentation with compound was characteristic of Nerolidol, with base peak 69 m/z and molecular ion of 204 m/z.

Betulinic acid: NMR ^{1}H (300 MHz, $CDCl_3$, TMS): (ppm) 0.66; 0.77; 0.90 e 0.96 (s, 5 X 3H; H23, H24, H25, H26, H27), 1.62 (s, 3H, H30), 2,97 (m, 1H, H3), 4.55 (sl, 1H, H29a), 4.68 (sl, 1H, H29b), 12.0 (sl 1H, H acid). NMR ^{13}C (75 MHz, $CDCl_3$, TMS): (ppm) 14.6 (C27), 15.3 (C24), 16.0 (C25), 16.1 (C26), 18.2 (C6), 19.3 (C30), 20.8 (C11), 25.5 (C12), 27.3 (C2), 27.9 (C23), 29.6 (C21), 30.5 (C15), 32.1 (C16), 34.3 (C7), 37.0 (C22), 37.1 (C10), 38.3 (C13), 38.7 (C1), 38.8 (C4), 40.6 (C8), 42.4 (C14), 46.9 (C18), 49.2 (C19), 50.52 (C9), 55.3 (C5), 56.2 (C17), 78.9 (C3), 109.6 (C29), 150.5 (C20), 179.3 (C28).

Glutinol: NMR ^{1}H (300 MHz, $CDCl_3$, TMS): ($CDCl_3$, 300 MHz) δ 5.63 (1H, br d, J = 5,7 Hz, H-6), 3.46 (1H, d, J = 32,7Hz, H-3) 1.16 (3H, s, H-28), 1.14 (3H, s, H-23), 1.09 (3H, s H-26), 1.04 (3H, s, H-24), 1.00 (3H, s, H-27), 0.99 (3H, s, H-30), 0.95 (3H, s, H-29), 0.85 (3H, s, H-25). NMR ^{13}C (75 MHz, $CDCl_3$, TMS): (ppm) 16.2 (C25), 18.2 (C2), 18.4 (C26), 19.6 (C27), 23.6 (C1), 25.4 (C-4), 27.8 (C7), 28.2 (C20), 28.9 (C23), 30.0 (C17), 30.3 (C12), 31.5 (C29), 32.0 (C15), 32.3 (C28), 33.1 (C21), 34.5 (C30), 34.6 (C11), 34.8 (C9), 35.0 (C19), 36.0 (C16), 37.8 (C13), 38.9 (C22), 39.3 (C4), 40.8 (C14), 43.0 (C8), 47.4 (C18), 49.6 (C10), 76.3 (C3), 122.0 (C6), 141.6 (C5).

Biological activity

Antimycoplasma activity

The antimollicute assays were collected from the Laboratory of Clinical Microbiology from FURB that provided the bacterial strains. Tests were evaluated against mollicutes strains (no-cell-wall bacteria) *Mycoplasma mycoides* subsp. *capri* (NCTC 10137). *Mycoplasma genitalium* (ATCC 33530), *Mycoplasma hominis (ATCC 23114), Mycoplasma subs capricolum (ATCC 27343), Mycoplasma pneumonia 129* (ATCC 13883), and *Mycoplasma pneumonia FH* (ATCC 13883) and were also assessed. For the growth of bacterial strain, broth MLA was used for *M. hominis,* SP4 broth for *M. mycoides* subsp. *capri* and *M. genitalium, M. subs capricolum, M. pneumonia 129* and *M. pneumonia FH* (Velleca et al., 1980).

The crude extracts and fractions from *E. handroana, E. brevistyla, E. catharinae* and *E. stigmatosa* were evaluated by determination of the minimum inhibitory concentration (MIC). The microdilution broth assay was performed in sterile 96-well microplates, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2012) for cell-wall bacteria and Bebear and Roberteson (1996) for mollicutes.

The samples were properly prepared and transferred to each microplate well with the appropriate culture medium, in order to

Table 1. Data on the plant material of the species studied.

Plant species	Place of collection	Collection season	Voucher number
E. brevistyla	Blumenau, SC, Brazil	April, 2015	FURB 13688
E. handroana	Blumenau, SC, Brazil	April, 2015	FURB 14108
E. catharinae	Itajaí, SC, Brazil	April, 2016	VCFilho 160
E. stigmatosa	Itajaí, SC, Brazil	August 2016	VCFilho 161

Table 2. Percent (%) solvent extraction of crude/fraction from the four *Eugenia* species

Extracts/Fractions	*E. handroana*	*E. brevistyla*	*E. catharinae*	*E. stigmatosa*
Hexane leaf extract	4.35	10.97	5.35	19.15
Chloroform/ DCM leaf fraction	13.24	3.28	18.44	6.51
Ethyl acetate leaf fraction	7.04	2.33	15.88	0.2
Hexane stem extract	1.29	3.9	9.98	1.18
Chloroform/ DCM stem fraction	21.83	4.92	27.8	19.2
Ethyl acetate stem fraction	10.13	8.38	20.19	24.2

Table 3. Yield of isolated compound from the four *Eugenia* species.

Isolated compound	Yeld (mg)
α,β-amirin	12.9 (FDCM-F EB) 799 (FINF-EB) 94.3 (FDCMF-EH)
Nerolidol	12.4
Betulinic Acid	42.0
Glutinol	23.2

obtain a two-fold serial dilution of the original extract in a 10% medium/dimethyl sulfoxide (DMSO) solution, obtaining sample concentrations ranging between 1000 to 7.81 µg.mL^{-1}. The inoculum containing 10^4 to 10^5 microorganisms per ml were then added to each well. A number of wells were reserved in each plate to test for sterility control (no inoculum added), positive control (gentamycin or ciprofloxacin), inoculum viability (no extract added), and the DMSO inhibitory effect. The microplates were incubated at 37°C ± 1°C for 24 or 48 h (depending on the bacterium). Thereafter, growth of mollicutes strains was detected by observing the colour change in the medium. The MIC was defined as the lowest concentration of the samples able to inhibit bacterial growth.

Antibacterial activity with cell wall bacterial and fungal

The antibacterial assays were conducted at the Laboratory of Clinical Microbiology from UNIVALI that provided the bacterial strains. The determination of the minimum inhibitory concentration (MIC) was performed by broth microdilution. The method consisted in preparing successive dilutions of the tested extracts (1000 µg/mL up to 2 µg/mL) in culture media Mueller-Hinton broth for the bacteria (*Staphylococcus aureus* and *Escherichia coli*) and Sabouraud broth for the yeast (*Candida albicans*). The media were inoculated with the microorganism under study, incubated and later verified the lowest concentration that inhibited its growth.

RESULTS AND DISCUSSION

Chromatographic fractionation of leaves and stems fractions of the four studied plants led to isolation and identification of the compounds shown in Tables 2 and 3. For the evaluation of the antibacterial activity, a criterion established by Holetz et al. (2002) was used. Samples with MIC values lower than 10 µg ml^{-1} were considered to have excellent antibacterial activity; between 10 and 100 µg ml^{-1} were considered good; values between 100 and 500 µg ml^{-1} were considered to be of moderate activity; values between 500 and 1000 µg ml^{-1} of low activity, and for MIC values higher than 1000 µg ml^{-1}, samples were considered inactive for the extracts and fractions. The isolated compounds were considered inactive with MIC higher than 100 µg ml^{-1}. The results for MIC of all the samples are shown in Table 4 (*Mycoplasma* strains) and Table 5 (cell wall bacterial and fungal strains).

Of all samples tested, the highest activity was observed against *Staphylococcus aureus. S. aureus*, a Gram-positive bacterium is responsible for a large number of infections ranging from simple infections, such as acne or cellulitis to severe infections like pneumonia, meningitis, endocarditis, toxic shock syndrome, and sepsis. This is particularly true for the infections caused by methicillin-resistant *S. aureus* (MRSA), which is most often resistant to multiple antibiotic classes and is responsible for the majority of infections (Boucher et al., 2010; Lin et al., 2013; Bolt et al., 2017).

The FAE-F of *E. handroana* (FAEF-EH) and the FAE-F of *E. brevistyla* (FAEF-EB) exhibited pronounced

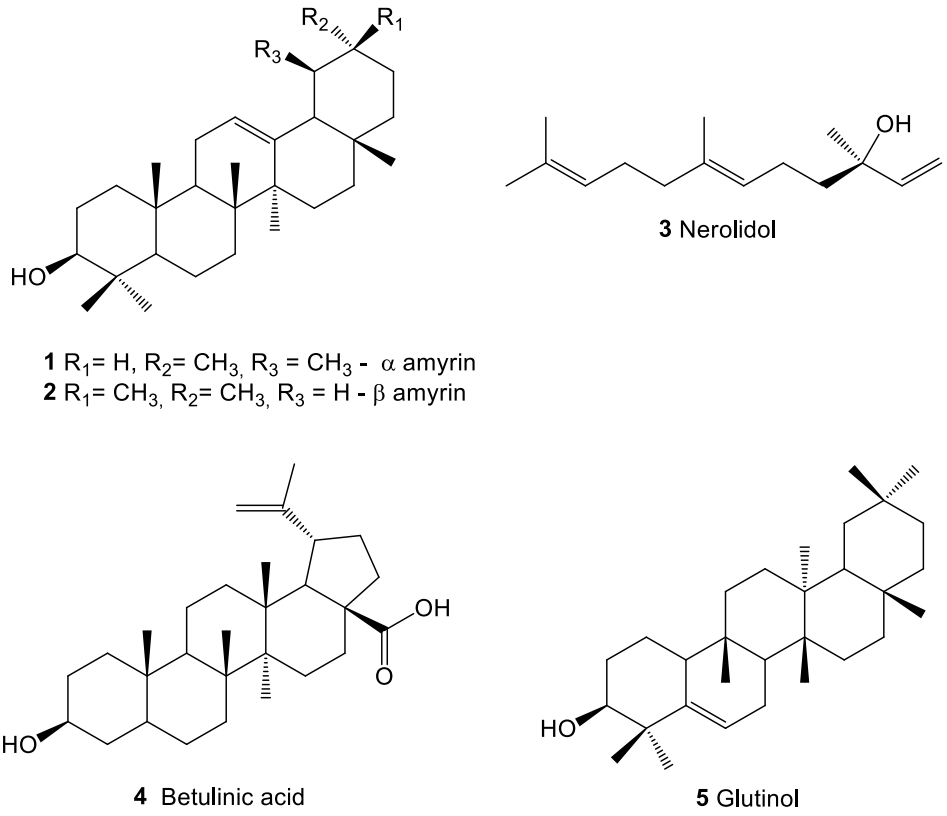

Figure 1. Chemical structure of the isolated compounds from the four Eugenia species studied.

antibacterial activity against *S. aureus* (MIC, 15.62 µg ml^{-1}). From the FAEF-EH, a mixture of α and β-amyrin which is known to have an important anti-bacterial activity against *S. aureus* (Jain et al., 2001) was isolated. Other fractions such as the EBMC-EB, FAEC-EB, EBMF-EB, FAEC-EH and EBMF-EH showed good results against *S. aureus* (MIC, 31.25 µg ml^{-1}). The results observed for *E. handroana* and *E. brevistyla* were the most promising. Antibacterial activity against *S. aureus* by other species of *Eugenia* such as *E. caryophyllata* and *E. brasiliensis* had been reported (Magina et al., 2012; Prakash et al., 2012). It is interesting to note that the fractions with higher polarity (ethyl acetate) showed better activity. On the other hand, the Gram-negative bacteria such as *E. coli* were not sensitive to the extracts and fractions until 1000 µg ml^{-1}.

Antifungal evaluation against *Candida albicans* showed that the extract and fractions from the stem of *E. stigmatosa* had the best activity with MIC of 125 µg ml^{-1} for the EBM-C and 250 µg ml^{-1} for FAEC- ES. The EMBC of *E. catharinae* also inhibited fungal growth at MIC = 250 µg ml$^{-1.}$ Some *Eugenia* species as *E. calycina* (Ferreira et al., 2014), *E. caryophyllata* (Mansourian et al., 2014), and *E. jambolana* (Satish et al., 2008) have been reported to have antifungal activity.

Among the tested samples, the highest activity against the *Mycoplasma* was observed in the FAEF-EH fraction against *M. pneumoniae* M129 (62.5 µg ml^{-1}). However, despite this pronounced activity, for the other species of *Mycoplasma*, the samples that presented the best activity were nonpolar fractions. $FCHCl_3F$-EB and FDCMC-EC showed MIC of 125 µg ml^{-1} against some samples tested. It is likely that highest presence of nonpolar compounds such as fatty acids and triterpenes in high quantity favored the action against the *Mycoplasma* strains.

Previous study by Tenfen et al. (2017) has attributed antimycoplasma activity of *E. platysema* to the presence of triterpenes. Another study by Zatelli et al. (2015) attributed anti-Mycoplasma activity of *E. hiemalis* to the essential oil component of the species. The isolated compounds in this study such as nerolidol **(3)** and the mixture of α,β amyrin **(1,2)** were inactive against the *Mycoplasma* strains. On the other hand, the compounds betulinic acid **(4)** and glutinol **(5)** showed encouraging activity against some strains tested.

Betulinic acid **(4)**, was considered inactive against strains of S*. aureus, E. coli* and *C. albicans* (MIC > 128 µg ml^{-1}) (Woldemichae et al., 2003), and considered active against some strains of *Mycoplasma* tested in this study, especially against *M. pneumoniae* FH, with MIC of

Table 4. Anti-*Mycoplasma* activity of some *Eugenia species*.

Samples	MIC (µg/mL^{-1})					
	M. hominis	***M. capricolum* subs. *capricolum***	***M. mycoides subsp. capri***	***M. genitalium***	***M. pneumoniae 129***	***M. pneumonie FH***
Eugenia brevistyla						
EBM-C	1000	500	500	500	250	250
FCHCl3-C	250	250	125	125	125	125
FAE-C	1000	1000	500	500	500	500
EBM-F	1000	1000	1000	1000	1000	500
FCHCl3-F	125	250	125	125	125	125
FAE-F	1000	1000	500	1000	250	1000
Eugenia catharinae						
EBM-C	500	500	500	500	NT	500
FDCM-C	125	125	125	125	NT	250
FAE-C	>1000	1000	>1000	1000	NT	>1000
EBM-F	500	500	250	250	NT	500
FDCM-F	250	250	250	250	NT	250
FAE-F	125	125	125	250	NT	125
Eugenia handroana						
EBM-C						
FDCM-C	250	250	125	125	125	125
FAE-C	>1000	>1000	>1000	>1000	>1000	>1000
EBM-F	>1000	>1000	>1000	>1000	>1000	>1000
FCHCl3-F	500	500	250	125	250	250
FAE-F	125	250	250	125	62.5	125
Eugenia stigmatosa						
EBM-C	500	250	1000	500	NT	250
FDCM-C	1000	1000	1000	1000	NT	500
FAE-C	500	250	500	500	NT	125
EBM -F	1000	1000	1000	1000	NT	500
FDCM-F	500	1000	500	500	NT	125
FAE-F	1000	1000	1000	1000	NT	500
Isolated compounds						
α ,β- amyrin	500	500	250	125	250	250
Nerolidol	>1000	>1000	1000	>1000	NT	>1000
Betulinic acid	>100	>100	100	100	NT	125
Glutinol	NT	100	100	100	NT	50
Cont. + (AZT)	2	2	2	2	2	2

12.5 µg ml^{-1}, demonstrating selectivity for this species. The *M. pneumoniae* FH is responsible for important diseases such as pneumonias, mainly in immunocompromised patients. It has a genetic structure different from the other species being considered more sensitive. On the other hand, glutinol **(5)** demonstrates antiviral, antifungal activity and potent anti-inflammatory activity as previously described (Madureira et al., 2003). Although some studies correlate the presence of this compound with antibacterial activity, there are no studies with this compound isolated against strains of *Mycoplasma.*

It is well-known that nerolidol **(3)** exhibits moderate antibacterial activity against *S. aureus.* However, its mechanism of action is related to intracellular K + leakage through the interaction of the carbonic chain of the molecule with the bacterial cell wall (Inoue et al., 2004). Since *Mycoplasma* do not have cell walls, they are

Table 5. Antibacterial activity against cell wall bacterial and fungal strains.

Samples	MIC (µg/ml)		
	Cell wall bacteria		Fungal strain
	S. aureus	*E. coli*	*C. albicans*
Eugenia brevistyla			
EBM-C	31.25	>1000	1000
FCHCl3-C	>1000	>1000	1000
FAE-C	31.25	>1000	500
EBM-F	31.25	1000	1000
FCHCl3-F	62.5	>1000	500
FAE-F	15.62	1000	500
E. handroana			
EBM-C	62.5	>1000	250
FDCM-C	>1000	>1000	>1000
FAE-C	62.5	>1000	1000
EBM-F	NT	NT	NT
FDCM-F	62.5	>1000	1000
FAE-F	125	1000	1000
E. catharinae			
FDCM-C	1000	>1000	>1000
FAE-C	31.25	1000	500
EBM-F	31.5	1000	1000
FCHCl3-F	250	>1000	1000
FAE-F	15.62	1000	500
E. stigmatosa			
EBM-C	125	1000	125
FDCM-C	NT	NT	NT
FAE-C	62.5	1000	250
EBM -F	125	1000	500
FDCM-F	1000	>1000	>100
FAE-F	NT	NT	NT

naturally resistant to molecules that act by this mechanism of action (MIC > 1000 µg ml^{-1}). Several studies attribute antibacterial activity for α,β-amyrin **(1,2)** against *S. aureus*, *E. coli* and *C. albicans*, however the mechanisms of action has not yet been elucidated.

Regarding the general antimicrobial effects, all the four species studied presented interesting antibacterial and antifungal activity. It is important to emphasize that this is the first work done to evaluate the chemical composition and antimicrobial activity of *E. handroana, E. brevistyla, E. catharinae* and *E. stigmatosa*. It is also the first study to evaluate the anti-*Mycoplasma* activity of α,β amyrin, nerolidol, betulinic acid, and glutinol.

The results found in this study are important because some of the microorganisms used in the study are responsible for various diseases, such as pneumonia, mastitis, skin and soft tissues infections, osteomyelitis, endocarditis, vaginitis, urethritis, and pyelonephritis in humans (Boucher et al., 2010; Cordova et al., 2010). Reports of resistance of cell wall bacteria and *Mycoplasma* to conventional treatments have also increased (Yechouron et al., 1992; Ma et al., 2017) and studies are in progress to determine other active principles present in the most promising species such as *E. brevistyla* and *E. handroana.*

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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