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Full Length Research Paper

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

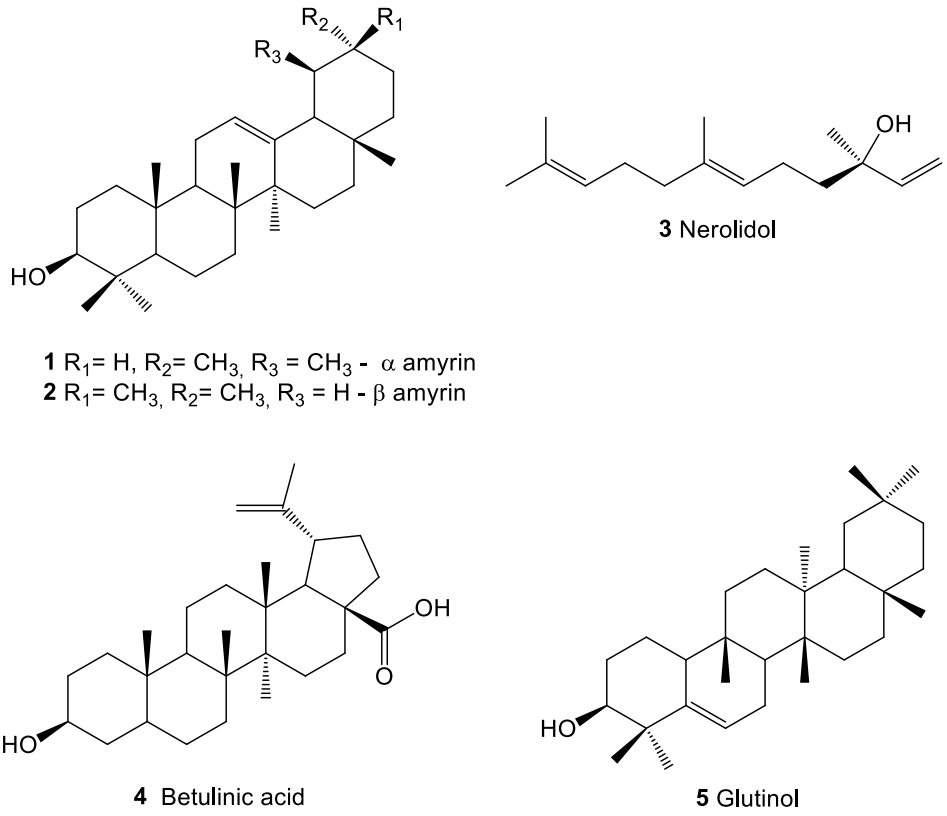

Figure 1. Chemical structure of the isolated compounds from the four Eugenia species studied.

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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Gastroprotective effect of the aqueous fraction of hydroacetonic leaf extract of *Eugenia uniflora* L. (Myrtaceae) (pitanga) against several gastric ulcer models in mice

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***Eugenia uniflora* L. (Myrtaceae) is popularly known in Brazil as pitanga or ibitanga. The infusion of *E. uniflora* leaves is being used in folk medicine as anti-diarrheal. The present study sought to evaluate the gastroprotective potential of the aqueous fraction of hydroacetonic leaf extract of pitanga (AFHP). The leaf powder of pitanga was extracted with 50% acetone using an overhead stirrer apparatus at room temperature in which the acetone was removed under reduced pressure and the suspended aqueous. The aqueous layer was freeze-dried to yield a 122 g aqueous fraction, which was stored at -20°C. Preliminary investigation showed that AFHP (100, 300 and 1000 mg/kg, p.o.) is devoid of any behavioral neurotoxic signs. The anti-ulcer activity of AFHP was evaluated in the gastric ulcer models induced by indomethacin, stress and HCl/EtOH in mice. In order to identify possible mechanisms of gastroprotective activity of AFHP, antisecretory activity of this fraction was conducted. The quantification of adhered gastric mucus reduced glutathione (GSH) and the role of nitric oxide (NO) were also investigated. The AFHP showed antiulcer activity in various models of acutely induced ulcers. The intra-duodenal administration of this fraction reduced total acidity and increased pH of the gastric secretion. Oral administration prevented a decrease in the amount of adhered mucus and increased GSH levels. Pretreatment with L-NAME did not affect the gastroprotective effect of AFHP. Our results suggest that AFHP exhibits antiulcer activity that involved an increased in gastric mucus and in the levels of GSH.**

Key words: *Eugenia uniflora*, pitanga, myrtaceae, gastric ulcer, gastroprotection, mucus.

INTRODUCTION

The peptic ulcer disease (PUD) is one of the most common disorders of gastrointestinal tract (TGI) with a prevalence of 4 to 5% in human society (El-Maraghy et al., 2015). The PUD is a gastrointestinal disorder that

occurs in the stomach and duodenum and generally is caused by an imbalance between aggressive and protective factors (Santin et al., 2010). It is well known that the major etiological factors involved in the onset of peptic ulcers are *Helicobacter pylori* infection, prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs), ischemia of the gastric mucosa, age, genetic factors, stress, alcohol, smoking and dietary habits (Caldas et al., 2011).

Medicinal plants are known as an important source of compounds for the treatment of gastric ulcers and new drugs discovery (Borrelli and Izzo, 2000; Zanatta et al., 2009). Several authors have shown that different species from Myrtaceae family such as *Campomanesia xanthocarpa* O. Berg (Markman et al., 2004), *Eugenia jambolana* (Chaturvedi et al., 2007; El-Shenawy, 2009), *Myrtus communis* L. (Sumbul et al., 2010), *Plinia edulis* (Vell.) Sobral (Ishikawa et al., 2008), *Eugenia dysenterica* DC. (Prado et al., 2014) and *Eugenia punicifolia (*Kunth) DC. (Basting et al., 2014) among others, have gastroprotective activity.

Eugenia uniflora L. (Myrtaceae) is popularly known as cherry, ibipitanga, pitanga or naganpiri (Consolini and Sarubbio, 2002; Rattmann et al., 2012; Weyerstahl et al., 1988). This species of bushy plant with edible fruit is native to Brazil and widely distributed in South America countries (Lorenzi and Souza, 1999). The fruits are rich in calcium, anthocyanins, flavonoids, carotenoids and vitamin C that conferred high antioxidant property of this species. The pleasant flavor and odor make this specie a desirable content of ice cream, juices, jams, wines and cosmetics (Lima et al., 2002; Lopes, 2008). The *E. uniflora* leaves are rich in tannins and flavonoids (Auricchio and Bacchi, 2003) and several studies have shown that tannin rich species have been traditionally used for their gastroprotective effects (de Jesus et al., 2012).

The folk medicine reports the use of hydro-alcoholic extract of *E. uniflora* leaves to control the levels of triglycerides, very low-density lipoproteins (VLDL) cholesterol and uric acid (Ferro et al., 1988). Furthermore, the use of *E. uniflora* leaves as anti-inflammatory, diuretic (Schapoval et al., 1994), antispasmodic (Amorim et al., 2009), antihypertensive (Consolini and Sarubbio, 2002), bactericidal, cytotoxic (Bouzada et al., 2009), anti-candida activity (Santos et al., 2013), anti-*Trypanosoma cruzi* activities (Santos et al., 2012) antidepressant-like effect (Victoria et al., 2013) and antidiarrheal (Almeida et al., 1995; Victoria et al., 2012) have also been reported. The objective of the present study was to evaluate the potential gastroprotective activity of the aqueous fraction of hydroacetonic leaf extract of the pitanga (AFHP).

MATERIALS AND METHODS

Botanical material

The *E. uniflora* leaves were collected at Anápolis, Goias, Brazil (16°20' 12.8 "S, 48°56' 19.3" W). The botanical material was authenticated by Prof. Dr. Heleno Ferreira Diaz of the Botany Department, Federal University of Goiás. A specimen voucher was deposited at the herbarium of the Federal University of Goiás (n°. 25477). The concentrations of AFHP and drugs were adjusted to ensure that all treatments respect the volume of 10 mL/kg in purified water for oral treatment or in saline for i.p administrations.

Preparation of aqueous fraction of hydroacetonic leaf extract

Dried and ground leaves of *E. uniflora* (1.0 kg) were exhaustively extracted with 50% acetone, using an overhead stirrer apparatus at room temperature. The acetone was removed under reduced pressure and the suspended aqueous extract was filtered to eliminate fats and chlorophylls. Following, a liquid-liquid extraction with ethyl acetate (10 × 150 mL) was carried out. The combined organic phase was evaporated to yield an ethyl acetate fraction (15 g). The aqueous layer was freeze-dried to yield a 122 g aqueous fraction, which was stored at -20°C.

Animals

Swiss albino male mice, weighing 25 to 35 g from the Central Animal Laboratory of the Federal University of Goiás were used. The animals were housed at 22±1°C on a 12 h (h) light/dark cycle with free access to food and water. All experiments were approved by the Ethics Committee on Animal Use (Protocol number 038/14).

Drugs and chemicals

The drugs and chemicals used included: ethanol (EtOH) (Quimex, São Paulo, SP, Brazil), carbenoxolone, L-Name (NG-nitro-L-arginine), indomethacin and Alcian blue (Sigma Chemical Company, St. Louis, MO, EUA); ranitidine (Teuto, Anápolis, GO, Brazil), sacarose (Lafan, Varzea Paulista, SP, Brazil), magnesium chloride (Quimibras, Rio de Janeiro, RJ, Brazil), sodium acetate (Vetec, Duque de Caxias, RJ, Brazil), sodium hydroxide (Lafan, Varzea Paulista, SP, Brazil), Ethylenediaminetetraacetic acid (EDTA), 5,5-ditiobis-2-nitrobenzoic acid (DTNB), trichloroacetic acid (TCA), and reduced glutathione (GSH).

General pharmacological activity test

The groups of animals (n = 3) were treated orally or intraperitoneally with increasing doses of AFHP (100, 300 or 1000 mg/kg), while the control group received vehicle (distilled water 10 mL/kg, p.o.) or saline (10 mL/kg, i.p.). After the treatments, the animals were observed for 3 min under free ambulation on the flat surface after 5, 10, 20, 30 and 60 min; 4, 8, 24 and 48 h; 4 and 7 days of treatment. The observed behavioral changes that differentiate treated animals of the control group were reported in standard pharmacological screening form (supplementary Table 1) adapted from Irwin proposal (Irwin, 1968).

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Indomethacin-induced ulcer

After 16 h of fasting, animals (n = 8/group) were orally treated with vehicle (10 mL/kg), AFHP (100, 300 and 1000 mg/kg) or ranitidine (50 mg/kg). After 60 min of treatment, all animals received indomethacin (30 mg/kg, s.c.) and after 3 h from the administration of the ulcerogenic agent, all treatments were repeated. Animals were euthanized 6 h after the administration of indomethacin to removed the stomachs for the evaluation of lesion index (LI) (Djahanguiri, 1969). The LI and the percentage of gastric ulcer inhibition were calculated according to Rios et al. (2010) (Table 1).

Hypothermic restraint stress ulcer

After 16 h of fasting, the animals (n = 8/group) received vehicle (10 mL/kg, p.o), AFHP (300 mg/kg, p.o) or ranitidine (50 mg/kg, p.o). One hour after treatment, gastric ulceration was induced by immobilizing the animals in a closed cylindrical cage maintained at 4°C. After 2 h, the mice were euthanized to remove the stomach for LI assessment (Senay and Levine, 1967).

Ethanol/HCl-induced ulcer

After 16 h of fasting, the animals (n = 8/group) received vehicle, AFHP (300 mg/kg) or carbenoxolone 200 mg/kg by gavage. One hour after treatment, all the animals received 0.3 M HCl/ethanol 60% solution (10 mL/kg, p.o.) orally to induce acute gastric lesions (Caldas et al., 2011).

The animals were euthanized 1 h after induction of gastric lesions, while the stomachs were removed and opened along the greater curvature. The stomachs were photographed and the area of lesions (%) was measured by AUTOCAD software.

Parameters involved in gastric acid secretion

The pylorus ligature was performed by adaptation of method described by Shay et al. (1945). After 16 h of fasting, animals (n=8/group) were anesthetized and pylorus ligature was carried out. Mice received vehicle, AFHP (300 mg/kg) or ranitidine (50 mg/kg) intraduodenally (i.d.). Four hours later the animals were sacrificed, the stomachs were removed and the gastric luminal contents were centrifuged for 30 min at 2000 *g.* The supernatant was used to measure the gastric juice volume (mL), total acidity and pH.

Quantification of gastric wall adhered mucus

The modified method of Corne et al. (1974) was used to quantify gastric mucus. After 16 h of fasting, the animals (n=8) received vehicle, AFHP (300 mg/kg) or carbenoxolone (200 mg/kg) by gavage. After 60 min, all groups were orally treated with 60% ethanol solution (10 mL/kg, p.o.). The contents of the stomach was weighed and transferred to a test tube containing 7 mL of 0.1% Alcian blue (0.16 M sucrose in 0.05 M sodium acetate, pH 5.8). After two consecutive rinses with 5 mL of sucrose (0.25 M), 5 mL of $MgCl_2$ (0.5 M) was added in each test tube for the extraction of mucus content with the dye. The glandular segment remained in this solution for 2 h with intermittent agitation. After which 4 mL of the resultant blue solution was agitated vigorously with 4 mL of ethyl ether until the formation of an emulsion and was centrifuged for 10 min at 3600 *g*. The absorbance of the supernatant was measured at 598 nm using a spectrophotometer. The concentration of Alcian blue was calculated through a calibration curve and the results were expressed in μg of Alcian blue/g of glandular tissue.

Quantification of reduced glutathione (GSH)

After ethanol induced gastric ulcer, the other half segment of the glandular stomach area was weighed and transferred to a tube where the homogenate was done with ice-cold 0.02 M ethylenediaminetetraacetic acid 10% (EDTA). According to the method proposed by Sedlak and Lindsay (1968), 400 µL aliquots of homogenate were mixed with 320 µL of distilled water and 80 µL of 50% trichloroacetic acid in Eppendorf tubes and centrifuged at 3000 g for 15 min. Subsequently, the supernatant (400 µL) was mixed with 800 µL Tris HCl (0.4 M, pH 8.9) and 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB 0.01 M). The absorbance was read within 3 min at 412 nm. The concentration of GSH was calculated using a standard curve of reduced glutathione (GSH) expressed in μg /g of tissue.

HCl/EtOH-induced gastric mucosa ulcer in mice pretreated with L-NAME

This method was performed as described by Matsuda et al. (1999). After a 16 h of fasting, the animals were pretreated with 0.9% saline (10 mL/kg, i.p.) or L-NAME, an inhibitor of NO synthase (20 mg/kg, i.p.). Thirty minutes later, animals received an oral dose of vehicle or AFHP (300 mg/kg). After 60 min, all groups were orally treated with 0.45M HCl/60% ethanol solution (10 mL/kg) to induce gastric-ulcer. After 1h, animals were euthanized and the stomachs were removed, opened along the greater curvature and gastric damage was determined as described above.

Statistical analysis

Results were expressed on means ± standard error of mean (SEM) absolute or percentage values and were compared using one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test (to analyze more than two groups) or student unpaired "t" test (to analyze two independent groups (Drummond and Tom, 2011a, b). Effects were considered significant at $p < 0.05$.

RESULTS

Effects of AFHP in the general pharmacological evaluation

AFHP at doses of 100, 300 and 1000 mg/kg administrated intraperitoneally (i.p.) caused a reduction of spontaneous movements and induced writhing which was observed between 5 min to 4 h. The dose of 100 mg/kg caused analgesia and alienation from 5 min until 4 h at a dose of 300 to 1000 mg/kg and in addition to the changes described above catatonia, ataxia, diarrhea were also observed. Death of animals was recorded at the highest dose (i.p) within 24 h. The animals treated with different doses by oral route showed no behavioral changes that differentiate animals treated with vehicle (supplementary Table 1).

Effect of AFHP in gastric ulcer induced by non-steroidal anti-inflammatory drug (NSAID)

The administration of indomethacin (NSAID) produced

Table 1. Score attribution scale for the degree of ulceration.

Index of lesion	Score
Discoloration of mucosa	1
Edema	1
Hemorrhages	1
Number of petechia	
Until 25%	2
More than 25%	3
Intensity of ulceration	
Ulcers or erosion up to 1 mm	*N* × 2
Ulcers or erosion larger than 1 mm	*N* × 3
Perforated ulcers	*N* × 4

N, Number of stomach lesions.

Table 2. Effects of AFHP or ranitidine on indomethacin induced ulcers in mice.

Group	Treatment (p.o.)	Dose (mg/kg)	LI	Reduction (%)
Control	Vehicle	-	8.7±0.7	-
Ranitidine	Ranitidine	50	5.1±0.3***	41.7
		100	5.9±0.5***	32.7
AFHP	AFHP	300	5.3 ±0.3***	38.7
		1000	5.0±0.5***	42.9

Results are expressed as mean ± SEM of the LI for eight mice. Statistical comparison was performed using ANOVA followed by the Tukey. ***p ≤ 0.001 compared with the control group.

extensive lesions in the gastric mucosa. Treatment of mice with AFHP (100, 300 or 1000 mg/kg) significantly reduced the LI (Table 2).

Effect of AFHP in gastric ulcer induced by hypothermic restraint stress

In the gastric ulcer induced by stress in the model of hypothermic restraint, treatment with AFHP or ranitidine significantly reduced the LI when compared with the control group (Table 3).

Effect of AFHP on gastric ulcer induced by HCl / ethanol in mice

Administration of HCl/ethanol yielded extensive lesions in the gastric mucosa of the stomach. These lesions were characterized by multiple red or dark brown spots of different sizes along the gastric mucosa (Figure 1). Treatment of mice with AFHP significantly reduced the ulcerated area by 58.9% when compared to the control group (Table 4).

Effect of AFHP on parameters of gastric acid secretion in mice

The treatment with AFHP (300 mg/kg) was unable to decrease the volume of gastric secretion. However, AFHP increased the pH and decreased the total acidity. The treatment with ranitidine (50 mg/kg) caused a decrease in the volume of gastric acid secretion, total acidity and increased pH (Table 5).

Effect of AFHP on gastric adhered mucus

The alcian blue binding capacity of gastric mucus in the control group with lesion (ethanol 60%, 10 mL/kg, p.o.) was significantly reduced compared with the control group without injury. However, the groups of animals with lesions that were pretreated with AFHP or carbenoxolone significantly increased the alcian blue binding capacity of gastric wall mucus (Table 6).

Effect of AFHP on the amount of GSH in the stomach tissue

The GSH content in the control group with lesion (EtOH

Table 3. Effects of AFHP or ranitidine on stress-induced gastric lesions in mice.

Group	Treatment (p.o.)	Dose (mg/kg)	Index of lesion	Reduction (%)
Control	Vehicle	-	9.1±1.0	-
Ranitidine	Ranitidine	50	5.1±0.3***	43.8
AFHP	AFHP	300	5.1±0.2***	43.8

Results are expressed as mean ± SEM of the LI for eight mice. Statistical comparison was performed using ANOVA followed by the Tukey. *** $p \leq 0.001$ compared with the control group.

Figure 1. Gastroprotective effects of AFHP or carbenoxolone on the HCl/ethanol-induced gastric lesion in mice.

Table 4. Effects of AFHP or carbenoxolone on HCl/ethanol-induced gastric lesions in mice.

Group	Treatment (p.o.)	Dose (mg/kg)	Ulcerated area (%)	Reduction (%)
Control	Vehicle	-	19.00±3.86	-
Carbenoxolone	Carbenoxolone	200	2.05±0.49***	89.2
AFHP	AFHP	300	7.80 ±2.3**	58.9

Results are expressed as mean ± SEM of the ulcerated area (%) for eight mice. Statistical comparison was performed using ANOVA followed by the Tukey ** $p \leq 0.01$ *** $p \leq 0.001$ compared with the control group.

Table 5. Effects of AFHP or ranitidine extract, administered intraduodenally, on the biochemical parameters of gastric juice obtained from pylorus ligature in mice.

Group	Treatment (i.d.)	Dose (mg/kg	Volume (ml)	pH	Gastric acidity (mEq[H^+] /L/4h)
Control	Vehicle	-	2.37±0.05	3.25±0.2	4.34±0.5
Ranitidine	Ranitidine	50	2.12±0.04*	3.69±0.05 **	1.79±0.07***
AFHP	AFHP	300	2.20±0.07	4.20±0.08***	2.40±0.3**

Results are expressed as mean ± SEM for eight mice. Statistical comparison was performed using ANOVA followed by the Tukey. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared with the control group.

60%, 10 mL/kg, p.o.) was significantly reduced compared with control group without injury. However, the groups of animals with lesions that were pretreated with AFHP or carbenoxolone significantly increased the GSH content in 48.24 or 11.84%, respectively, when compared with control group with lesion (Table 6).

Effect of AFHP on HCl/ethanol-induced gastric mucosal lesion with L-NAME-pretreated mice

Treatment with HCl/EtOH induced extensive lesions in the gastric mucosa of the stomach. However, treatment with AFHP significantly reduced the ulcerated area in

Table 6. Effect of oral treatment of AFHP or carbenoxolone on the gastric adhered mucus and GSH in the model of ethanol (60 %, 10 mL/kg, p.o.) in mice.

Group	Treatment (p.o.)	Dose (mg/kg)	Alcian blue (µg/g tissue)	GSH (µg/g tissue
Control without lesion	Vehicle + water	-	38.4±2.3	150.4±5.6
Control with lesion	Vehicle + EtOH	-	29.1±2.1*	128.0±3.0**
Carbenoxolone	Carbenoxolone + EtOH	200	41.0±1.4$^{\#\#}$	143.5±3.8$^{\#}$
AFHP	AFHP + EtOH	300	37.5±1.7$^{\#}$	190.2±11.8$^{\#\#}$

Results are expressed as mean ± SEM for eight mice. Statistical comparison was performed using ANOVA followed by the Tukey test. * $p \leq 0.05$, ** $p \leq 0.01$, control without lesion vs control with lesion; $^{\#}$ $p \leq 0.05$, $^{\#\#}$ $p \leq 0.01$, carbenoxolone or AFHP vs control with lesion.

Table 7. Role of nitric oxide (NO) in the gastroprotective effect of AFHP against HCl/EtOH-induced gastric injury in mice.

Pretreatment (i.p.)	Treatment (p.o.)	Ulcerated Area (%)
Saline 10 mL/kg	Vehicle 10 mL/kg	64.8±3.3
Saline 10 mL/kg	AFHP 300 mg/kg	23.2±1.9***
L-Name 20 mg/kg	Vehicle 10 mL/kg	68.1±5.0
L-Name 20 mg/kg	AFHP 300 mg/kg	16.5±3.5***

Results are expressed as mean ± SEM for eight mice. Statistical comparison was performed using ANOVA followed by the Tukey test.. *** $p \leq 0.001$ compared with the control group (treated with saline + vehicle).

64.2%. Pretreatment with L-NAME did not reverse the gastroprotective activity of AFHP (Table 7).

DISCUSSION

Due to the series of side effects associated with the first line of antiulcer drugs, the study of medicinal plants derived compounds for the treatment of various gastrointestinal disorders is becoming important around the world (Zheng et al., 2014). The phytochemical study of *Eugenia uniflora* allowed the isolation and structure elucidation of several phenolic substances of three types: galoil esters, ellagitannins monomeric and dimeric flavonoids and glycosides. Among the compounds identified in the aqueous fraction of hidroacetonic extract of *E. uniflora* leaves are: oenothein B, myricitrin, quercitrin, eugeniflorin D2 and camptothin A (Fortes et al., 2015).

The present study evaluated the gastroprotective effect of aqueous fraction of hydroacetonic leaf extract of pitanga (AFHP) in different models of experimentally induced gastric ulcers and the possible mechanisms of actions involved in this effect. According to Parmar and Desai (1993) various mechanisms in different experimental models of gastric ulcers make it impossible to think of a single mechanism of gastroprotective activity. Recent studies have shown that the leaf extract of *E. dysenterica* and *E. punicifolia* have gastro-protective activity (Basting et al., 2014; Prado et al., 2014).

An important factor involved in the pathogenesis of gastric lesions induced by anti-inflammatory nonsteroidal drugs (NSAIDs) is a deficiency of endogenous prostaglandins (PGs). Prostaglandins, particularly PGE_2 and PGI_2 are described as key mediators in gastric mucosal defense. Its cytoprotective effect has been associated with the stimulation of mucus/bicarbonate secretion, maintenance of mucosal blood flow and inhibition of gastric acid secretion (Martins et al., 2014; Sousa et al., 2013).

The indomethacin (NSAID) inhibits the production of prostaglandins leading to a decrease of the formation and release of mucus/bicarbonate and increases the production of HCl, thereby favoring the appearance of gastrointestinal ulcers (Wallace and Devchand, 2005). Our data suggest that oral treatment with AFHP (100, 300 and 1000 mg/kg), ranitidine (50 mg/kg) protected the gastric mucosal lesions induced by NSAIDs. These results suggest possible involvement of mucus and or PGs.

The stress induced ulcers occur as a result of stressful events such as, burns, sepsis, surgery and trauma (de Almeida et al., 2012). Several studies show that disturbances in gastric secretion, changes in microcirculation and abnormal gastric motility are the possible mechanisms involved in stress-induced ulcers (Amany and Ibrahim, 2013; Batista et al., 2004). Stress reduces endogenous glutathione levels and promotes generation of reactive species such as $OH^{\cdot}$ and inhibit the biosynthesis of mucosal prostaglandins by H_2O_2

accumulation (Bandyopadhyay et al., 2002). The cold restraint stress model (4°C, 2 h) has been widely used to evaluate anti-ulcer activity (Viana et al., 2013). The results suggest that the gastroprotective activity AFHP in stress -induced gastric injury is probably mediated by its antisecretory and antioxidant activity. GSH is a major mediator involved in maintaining the integrity of the gastric mucosa, due to its antioxidant capacity (Mutoh et al., 1990). It is known that gastric lesions induced by stress and EtOH are associated with significant decrease in mucosal levels of GSH (Szabo and Vattay, 1990). The administration of AFHP significantly increased the GSH levels in gastric mucosa. This result suggests that AFHP has antioxidant activity.

The effect of AFHP was also evaluated in ulcer model induced by HCl/EtOH. It is known that the ingestion of large amounts of ethanol promotes gastric lesions in humans and experimental animals (Siegmund et al., 2003; Teyssen and Singer, 2003). It is well established that the formation of gastric mucosal lesions by necrotizing agents such as HCl/EtOH enhances lesions and reduce the number of gastric defense mechanisms such as disruption of blood flow, degranulation of mast cells, reduction of prostaglandin and mucus/bicarbonate release (Abdel-Salam et al., 2001; Glavin and Szabo, 1992). Oral administration of HCl / EtOH in the control group promoted necrotic lesions and bleeding characteristics. Based on the results obtained from this model, AFHP reduced lesion area. An increase in prostaglandin synthesis or mucus adhered to gastric mucosa may be one of the responsible for the antiulcerogenic mechanisms. These results are in agreement with those found by Viana et al. (2013). Mucus is one of the most important parameters that contribute to the protection of gastric mucosa. Gastric mucus is responsible for the first line of defense of the gastric mucosa and consists of a transparent viscous, elastic, adherent gel that is made up of water and glycoproteins (Martins et al., 2015). The mucus layer is a physical barrier that adheres together with bicarbonate and protects the underlying mucosa from proteolytic digestion (Allen and Flemstrom, 2005). When the mucus barrier is damaged, the gastric mucosa becomes more susceptible to gastric acid induced ulcers (Santin et al., 2010). This study revealed that the amount of adhered gastric mucus was increased by treatment with AFHP and this increase probably contributed to the cytoprotective effect of AFHP. Pyloric ligation model produces biochemical parameters in gastric mucosa such as volume, pH and total acidity. The pyloric ligation interferes with gastric mucosa and changes in the levels of prostaglandins, cytokines and endogenous glutathione (Singh et al., 2008).

In an attempt to determine the gastroprotection mechanisms of AFHP, the parameters of gastric acid secretion were evaluated. We found that intraduodenal administration of AFHP significantly reduced total acidity and increased pH. This result indicates a systemic action in addition to anti-secretory activity of AFHP. The reference drug, ranitidine (50 mg/kg), significantly reduced the volume of gastric juice, total acidity and increased the pH.

It is known that nitric oxide (NO) plays an important role in the defense of the gastric mucosa and is a biological mediator which regulates the secretion of mucus and blood flow (Falcao Hde et al., 2013). In the gastrointestinal tract, NO is also involved in modulating the activity of mast cells together with endogenous prostaglandins (Klein-Junior et al., 2013; Wallace, 2006). Our results showed that the inhibition of NO synthase by L-NAME did not reverse gastroprotection effect of AFHP, thereby suggesting that NO synthesis is not critical to its gastroprotective activity.

Conclusion

Overall findings in the current study showed the effectiveness of AFHP in preventing gastric ulcers against lesions induced in various experimental models. These effects could be associated with the gastric cytoprotective mechanisms including the participation of mucus and GSH levels on the mucosa. Further studies will be focused to elucidate the phytochemical(s) responsible(s) for the antiulcer mechanism of AFHP.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Table 1.

Route of administration	Dose (mg/kg)	Observed changes
Intraperitoneal	100	Reduction of spontaneous movement (up until 4 hrs), analgesia and alienation (5, 10, 20, 30 and 60 min)
	300	Reduction of spontaneous movement (up to 4 h), catatonia, analgesia, ataxia, alienation, diarrhea, contortion (up to 4 h) and death within 24 h
	1000	Reduction of spontaneous movement (up to 4 h), catatonia, analgesia, ataxia, alienation, diarrhea, contortion (up to 4 h) and death within 24 h
Oral		No changes observed.

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Full Length Research Paper

Immunoregulatory activity of root bark of *Cassia sieberiana* D.C. in a modified adjuvant-induced arthritis in rat

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The present study seeks to evaluate the immunoregulatory effects of extracts of the root bark of *Cassia sieberiana,* a plant used in Ghana for various painful inflammatory conditions, in a modified adjuvant arthritis model induced by administration of very low dose of *Mycobacterium tuberculosis* (MT) – carrageenan mixture in the rat. A volume of 0.1 mg kg^{-1} heat killed MT in paraffin oil was mixed with equal volume of 0.05% (w/v, normal saline) carrageenan. A single intraplantar dose of 0.1 ml of the MT - carrageenan mixture was administered to experimental animals. Groups were administered extracts (20 to 200 mg kg^{-1}, p.o.), dexamethasone (0.3 mg kg^{-1}, p.o.) or vehicle an hour prior to the test and daily from test day till the 6th day. Paw volume (ml) of the injected hind limbs were measured using a plethysmometer, while paw withdrawal thresholds were determined using an analgesy meter. Serum levels of IL-1α, IL-6, IL-10 and TNF-α were determined via enzyme linked immunosorbent assay (ELISA). Results showed that the extracts attenuated the inflammation and hyperalgesia caused by the intraplantar injection of MT-carrageenan mixture in the rats in a dose-dependent fashion. Similarly, the extracts reduced the serum levels of IL-1α, IL-6 and TNF- α while increasing the levels of IL-10. It can be concluded that the anti-inflammatory activity of extracts of root bark of *C. sieberiana* may be attributable to their immunomodulatory effects via suppression of pro-inflammatory cytokines, TNF-α, IL-1α and IL-6; and elevation of the anti-inflammatory cytokine, IL-10 levels, in serum.

Key words: Immunoregulatory, pro-inflammatory cytokines, TNF-α, adjuvant, *C. sieberiana*, IL-10.

INTRODUCTION

Cassia sieberiana is a woody shrub which grows well in tree or shrub savanna with less than 800 mm annual rainfall (Von Maydell, 1990), and it is a tropical plant which is native to Africa. The plant has many uses including food, medicine and other unspecific uses (Freedman, 2012). It has many folkloric uses including

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the root bark being used for treating swelling, gout and dropsy, and leaves used to treat rheumatism and arthritis (Obidah et al., 2009; Madusolumuo et al., 1999). Earlier, evaluations on the anti-inflammatory effects of the aqueous and ethyl acetate fractions of ethanolic extract of the root barks of *C. sieberiana* in the carrageenan-induced edema and formalin tests have been conducted (Donkor et al., 2013). In this follow up study, its immunoregulatory effect is studied as a possible mechanism responsible for its anti-inflammatory activity.

Inflammation is a localized protective reaction of cells/ tissues of the body to allergic or chemical irritation, injury and/or infections. It is characterized by pain, heat, redness, swelling and loss of function that result from dilation of the blood vessels leading to an increased blood supply and increased intercellular spaces resulting in the movement of leukocytes, protein and fluids into the injured regions (Parham, 2000). It is evident that the immune system is intricately linked to the etiologic and pathophysiologic mechanisms of inflammation (Chun et al., 2016). It is known that some cytokines (IL-3-4,-5,-10,-13) released during inflammation are beneficial by acting as anti-inflammatory mediator within the cells while pro-inflammatory mediators present pathways through which disorders in the body may be eliminated (Esch and Stefano, 2002).

Indeed studies suggest that pro-inflammatory cytokines have to be taken care of in order to completely overcome the effect of inflammatory responses (Meshram et al., 2016). Medicinal plants are rich sources of substances which are claimed to induce non-specific immunomodulatory effects (Sharififar et al., 2009).

Complete Freud's Adjuvant (CFA)-induced arthritis is the commonest chronic arthritic animal model. The main challenge associated with this assay is that the heat killed *Mycobacterium tuberculosis* used is quite expensive thus many resource limited labs are unable to acquire it. Therefore, the present study seeks to modify it with the aim of reducing the cost of carrying it out by administration of very low dose of *Mycobacterium tuberculosis* – carrageenan mixture in rat.

METHODOLOGY

Plant collection

The roots of *C. sieberiana* was obtained from the Arboretum of the Centre for Plant Medicine Research (CPMR), Mampong-Akwapim, Ghana. The barks of the root was peeled and air-dried. The plant was authenticated by the Plant Development Department (PDD) of the CPMR and a voucher specimen (CSRPM No. 315) kept in the Herbarium of the PDD of the CPMR, Mampong-Akwapim, Ghana.

Extract preparation

The dried root barks were crushed in a mortar, and 2.0 kg was soaked in 4 L of absolute ethanol for 3 days. The mixture was filtered, and the filtrate dried at 65°C under pressure using rotary evaporator (Eyela, N-1100, Rikakikai Co. Ltd, Tokyo, Japan). The extract was defatted using 350 ml of petroleum ether. The filtrate was mixed with 350 ml of 90% ethanol and the ethanol was evaporated under pressure at 65°C. The resultant aqueous filtrate was mixed with 250 ml distilled water. A volume of 250 ml of ethyl acetate was used to partition the residue three times, and a freeze dryer used to lyophilize the aqueous layer to dry powder; the aqueous extract was labeled as CS-Aq. The ethyl acetate was evaporated under pressure as previously described to a paste, lyophilized thereafter to dryness and labeled as ethyl acetate extract (CS-Ea).

Animals

Male Sprague-Dawley rats (180 to 200 g) were obtained from the Animal Unit, CPMR, Mampong-Akuapem, in the Eastern Region of Ghana. The animals were fed on feed obtained from Ghana Agro Food Company (GAFCO), Tema, Ghana. They were also allowed free access to clean water. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care (NIH, No.85 to 23, revised 1985). All protocol were approved by the Pharmacology/Toxicology Department of the CPMR with animal use authorization number (CPMR No. 12/15) kept at the repository of the department. The paper was written using the ARRIVE guidelines.

Chemicals and drugs

Dexamethasone and heat killed *M. tuberculosis* were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). TNF-α, IL-1α, IL-6 and IL-10 rat serum ELISA kits were obtained from Abcam Company Ltd (Cambridge, USA). All other chemicals were purchased in their purest form available from British Drug House (BDH) Ltd (Poole, UK).

Induction of FA-carrageenan-induced arthritis

In adjuvant-induced arthritis, heat killed *M. tuberculosis* in paraffin oil is normally administered at 4 to 10 mg kg^{-1} while carrageenan is usually administered (0.1-1%, w/v) in carrageenan-induced edema assay (Pearson, 1956). In an attempt to modify CFA-induce arthritis with the aim of reducing the cost involved, a volume of 0.1 mg kg^{-1} heat killed *M. tuberculosis* in paraffin oil was mixed with equal volume of 0.05% (w/v, normal saline) carrageenan. Right hind paw of rats were injected with a single intraplantar dose of 0.1 ml of the *M. tuberculosis* (MT) - carrageenan mixture. Groups were administered extracts (20, 100 and 200 mg kg^{-1}, p.o.), dexamethasone (0.3 mg kg^{-1}, p.o.) or vehicle an hour prior to the test, and daily from test day till the 6th day. On the day of test, paw volume (ml) of the right hind limbs were measured using a plethysmometer (7150, Ugo basile, Comerio-Varese, Italy), prior to the induction of arthritis (baseline) and thereafter readings were taken daily until the 6th day. The anti-inflammatory activity was calculated as the degree of paw edema (e) using the formula:

$$e = \frac{E_t - E_o}{E_o} \times 100\%$$

where, E_o and E_t are paw volume at baseline, and at a given reading day of the right hind paw.

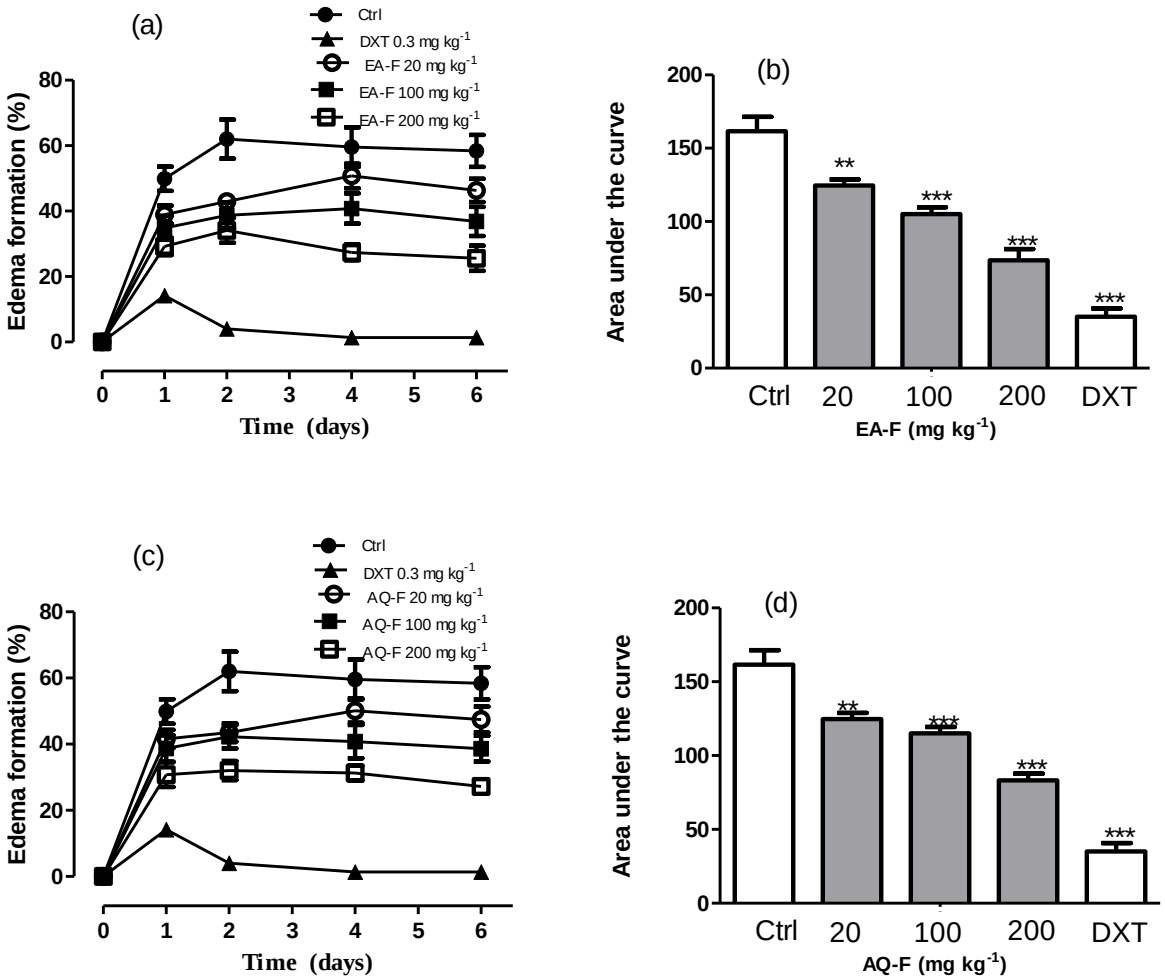

Figure 1. (a) Effect of EA-F (20-200 mg kg^{-1}, p.o.), (c) AQ-F (20-200 mg kg^{-1}, p.o.) and dexamethasone, DXT (0.3 mg kg^{-1}, p.o.) on the time course of MT-Carrageenan-induced arthritis in rats; (b) and (d) are AUCs determined from (a) and (c) respectively. Each point/column represents Mean±SEM (n = 5) (**p<0.01 and ***p<0.001 are compared to untreated controls).

Hyperalgesia determination

Paw withdrawal thresholds (PWTs) were determined using an analgesy-meter (7200, Ugo Basile, Comerio-Varese, Italy). The PWTs were measured prior to the induction of arthritis (baseline) ,and thereafter readings taken daily until the 6th day. The analgesic activity of the extracts/drug was calculated as analgesic coefficient (k) using the formula:

$$k = \frac{a + b + c + d + e + f}{y * 6} * 100\%$$

Where a to f are daily PWTs up to day 6, and y is baseline PWTs.

Determination of serum levels of TNF-α, IL-α, IL-6 and IL-10

On day 6 (termination of treatment), blood samples were collected via tail bleeding, and serum prepared and stored at appropriate temperature for determination of TNF-α, IL-α, IL-6 and IL-10 levels via ELISA following the procedure of the manufacturers.

Statistical analysis

One way analysis of variance (ANOVA) and Bonferroni post-hoc tests were conducted between control and tests to determine statistical significance. Further comparisons between vehicle- and drug-treated groups were performed using the Newman - Keuls' Test. GraphPad Prism for Windows Version 5.00 (GraphPad Software, San Diego, CA, USA) was used for all graphics and statistical analyses. The 5% level of probability was used as criterion of significance in all instances.

RESULTS

FA-carrageenan-induced arthritis

The effects of the extracts and dexamethasone on MT-carrageenan-induced arthritis are represented in Figure 1. The sub-plantar injection of MT-carrageenan mixture caused an increase in paw volume of all experimental rats

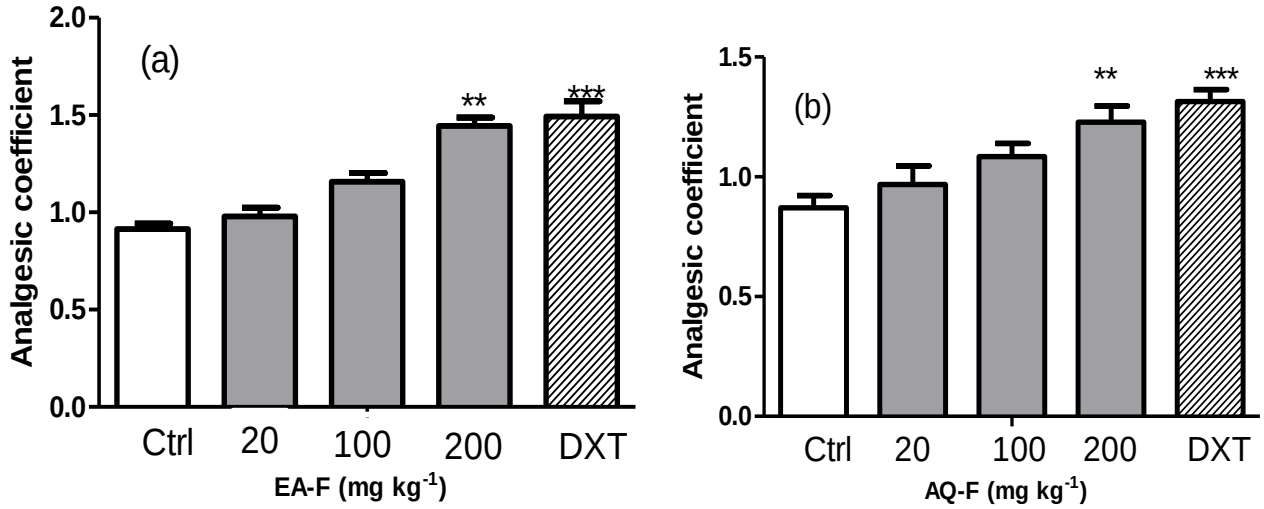

Figure 2. (a) Effect of Ea-F (20-200 mg kg^{-1}, p.o.), (b) AQ-F (20-200 mg kg^{-1}. p.o.) and dexamethasone, DXT (0.3 mg kg^{-1}, p.o.) on *M. tuberculosis* carrageenan-induced hyperalgesia in rats. Values are Mean±SEM (n = 5) (**p<0.01 and ***p<0.001 compared to untreated controls).

with controls experiencing a sustained increase in paw volume over the 6 day study period which peaked after a day post-arthritis induction. Pretreatment with dexamethasone (0.3 mg kg^{-1}, p.o.) completely reversed the edema after day 2 post-arthritis induction. Both extracts caused significant ($p < 0.001$) dose-dependent reduction in the percentage edema formation compared to the controls. The degree of edema inhibition over the treatment period calculated as area under the curve (AUC) for the ethyl acetate and aqueous fractions of the ethanolic extract (38.2 to 76.1%) was lower than dexamethasone (95.4%).

Mechanical hyperalgesia

Figure 2 shows the effects of the extract and dexamethasone on the MT-carrageenan-induced mechanical hyperalgesia following the sub-plantar injection of MT-carrageenan mixture. The ipsilateral paw showed marked hyperalgesia in all experimental rats after a day of injection of the MT-carrageenan which was reduced significantly in both extracts and reference drug ($p < 0.0001$) in a dose-dependent fashion compared to controls.

Involvement of TNF-α

The effects of the extracts (20 to 200 mg kg^{-1}, *p.o*) and dexamethasone (0.3 mg kg^{-1}, *p.o*) on the serum levels of TNF-α in MT-carrageenan-induced arthritis in the rat after 6 days are represented in Figure 3. Intraplantar injection of adjuvant caused an elevation in the serum TNF-α levels of rats which was attenuated by extracts and dexamethasone. Pretreatment with extracts significantly reduced serum TNF-α levels in both extracts-treated animals ($p<0.0001$) in a dose-related manner compared to controls.

Involvement of IL-1α and IL-6

The effects of the extracts (20 to 200 mg kg^{-1}, p.o) and dexamethasone (0.3 mg kg^{-1}, p.o) on the serum levels of IL-1α and IL-6 in MT-carrageenan-induced arthritis in the rat after 6 days are presented in Figures 4 and 5. Intraplantar injection of adjuvant caused an elevation in the serum IL-1α and IL-6 levels of rats which were attenuated by extracts and dexamethasone. Pretreatment with extracts significantly reduced serum IL-1α levels in both extracts ($p < 0.0001$) in a dose-dependent manner compared to controls. Similarly, serum IL-6 levels were gaudily reduced in both extracts- treated ($p < 0.0001$) rats in a dose-related manner compared to controls.

Involvement of IL-10

The effects of the extracts (20 to 200 mg kg^{-1}, p.o) and dexamethasone (0.3 mg kg^{-1}, p.o) on the serum levels of IL-10 in MT-carrageenan-induced arthritis in the rat after 6 days are presented in Figure 6. Intraplantar injection of adjuvant caused a reduction in the serum IL-10 levels of rats. Pretreatment with extracts and dexamethasone significantly increased serum IL-10 levels in both extracts ($p < 0.0001$), in a dose-dependent manner compared to controls.

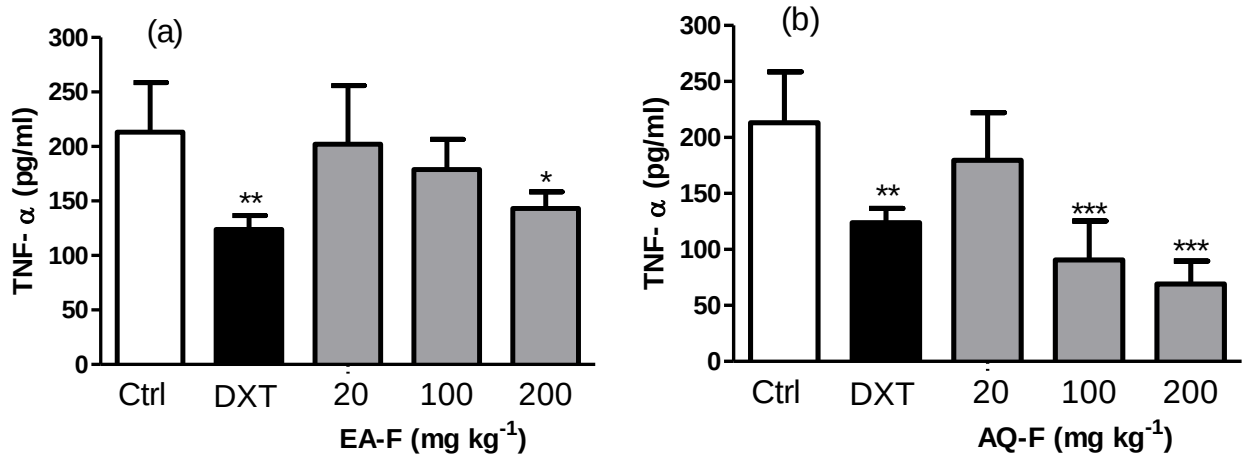

Figure 3. (a) Effect of EA-F (20-200 mg kg^{-1}. p.o.), (b) AQ-F (20-200 mg kg^{-1}. p.o.) and dexamethasone, DXT (0.3 mg kg^{-1}, p.o.) on serum TNF-α levels in MT-Carrageenan-induced arthritis in the rats after 6 days of treatment. Values are Mean±SEM (n = 5). *p<0.05, **p<0.01, and ***p<0.001 compared to untreated controls.

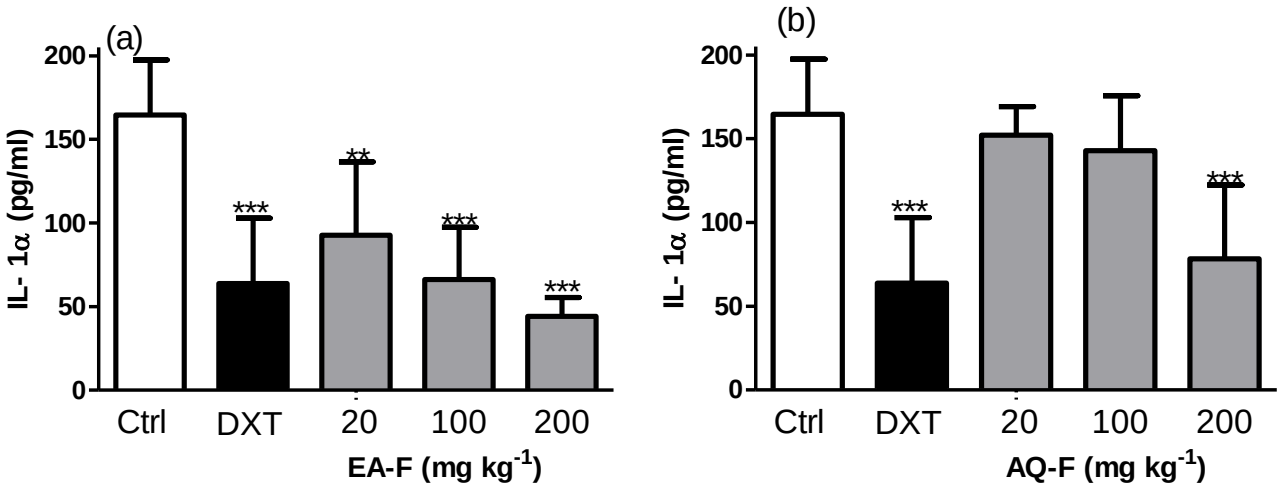

Figure 4. (a) Effect of EA-F (20-200 mg kg^{-1}. p.o.), (b) AQ-F (20-200 mg kg^{-1}. p.o.) and dexamethasone, DXT (0.3 mg kg^{-1}, p.o.) on serum IL-1α levels in MT-carrageenan-induced arthritis in the rats after 6 days of treatment. Values are Mean±SEM (n = 5). **p<0.01, and ***p<0.001 are compared to untreated controls.

DISCUSSION

It has been suggested that all pain, whether acute or chronic, peripheral or central, originates from inflammation and the inflammatory responses (Omoigui, 2007). Thus, the treatment of inflammation is a major clinical concern. We had earlier on established the anti-inflammatory effects of the root bark of *C. sieberiana* in both carrageenan-induced edema and formalin tests (Donkor et al., 2013).

A preliminary fractionation process of the crude ethanolic extract of root bark of the plant revealed that about 70% of the extract was in the ethyl acetate fraction. The root bark of the plant is traditionally boiled in water and drank. Thus, the present study sought to evaluate the immunoregulatory activity of ethyl acetate and aqueous fractions of ethanolic extract of the root bark of the plant as a possible mechanism for its anti-inflammatory properties. In this study, we have successfully induced arthritis using a single administration of very low dose mixture of Freud's adjuvant and carrageenan. The edema and hyperalgesia caused by the induction of the arthritis was attenuated by both fractions of the extract. The extracts also significantly reduced serum levels of pro-inflammatory cytokines, TNF-α, IL-1α and IL-6, while elevating levels of the anti-inflammatory cytokine IL-10.

A single intraplantar injection of 0.1 ml of *M.*

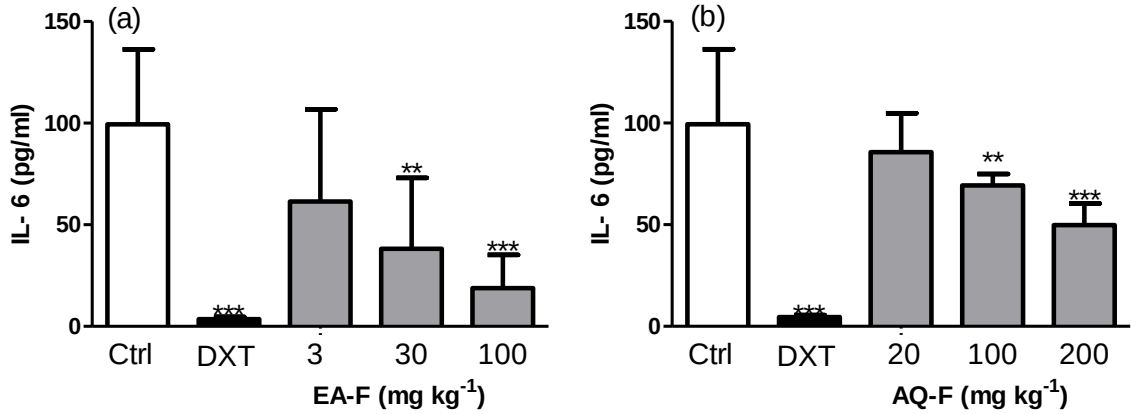

Figure 5. (a) Effect of EA-F (20-200 mg kg^{-1}. p.o.), (b) AQ-F (20-200 mg kg^{-1}. p.o.) and dexamethasone, DXT (0.3 mg kg^{-1}, p.o.) on serum IL-6 levels in MT carrageenan induced arthritis in the rats after 6 days of treatment. Values are Mean±SEM (n = 5). **p<0.01, and ***p<0.001 are compared to untreated controls.

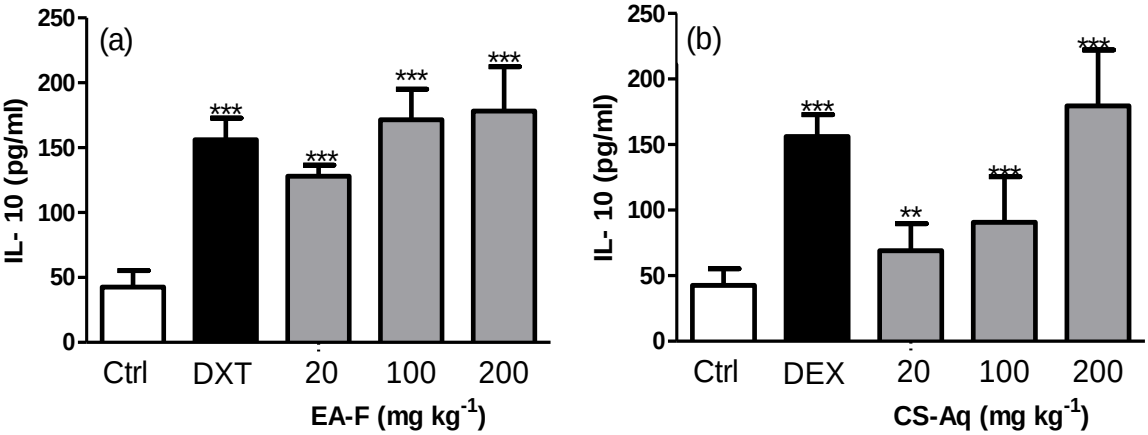

Figure 6. (a) Effect of EA-F (2-200 mg kg^{-1}. p.o.), (b) AQ-F (20-200 mg kg^{-1}. p.o.) and dexamethasone, DXT (0.3 mg kg^{-1}, p.o.) on serum IL-10 levels in MT carrageenan-induced arthritis in the rats after 6 days of treatment. Values are Mean±SEM (n = 5). **p<0.01, and ***p<0.001 are compared to untreated controls.

tuberculosis - carrageenan mixture induced inflammation in the rat which is identical in appearance to the arthritis induced by CFA. Like the arthritis induced by CFA, this one is also irreversible naturally saved by pharmacological intervention. Carrageenan-induced edema and the arthritis induced by Freud's adjuvant are known to be mediated by histamine, serotonin and kinin at the early phases, and they are sustained by prostaglandins and cytokines released from infiltrated leukocytes (Phillippe et al., 1997; Maleki et al., 2001; Okada et al., 2014).

Though the chemical nature of the current model of chronic inflammation is not yet characterized, it is strongly believed that the inflammatory mediators would be similar to that of carrageenan-induced inflammation and CFA-induced arthritis. The advantage of this model is that the reagents used in inducing the inflammation are low in concentration thus more tolerable and humane to the subjects. It would also be useful in a resource limited facility in terms of cost reduction.

It has been reported that infiltrated neutrophils-released prostaglandin E2 directly sensitizes mechanical nociceptors to produce hypernociception in carrageenan assay (Furst and Emery, 2014). It has also been shown that the inflammation causes a lowering of the thresholds of various mechanoreceptors and mechanotransduction pathways (Park et al., 2008; Liu et al., 2014).

Similarly, the production of pro-inflammatory cell-mediated cytokines such as IL-1α, IL-6 and TNF-α are considered as the main reason for hyperalgesia and

allodynia induction during acute and chronic inflammation situations (Lipsky, 2006; Chun et al., 2016). The ethyl acetate and aqueous fractions of the ethanolic extract together with dexamethasone significantly reduced the hyperalgesia caused by the injection of the *M. tuberculosis*-carrageenan mixture suggesting interference in the production and/or release of the associated inflammation mediators peripherally.

In the mechanical hyperalgesia test, it has been suggested that the stimulus applied is likely to activate slowly-adapting mechanoreceptors with decreased thresholds, which are predominantly C-fiber located in the cutaneous and subcutaneous structures that would have required greater stimulus intensities for activation (Birder and Perl, 1994; Lewin and Moshourab, 2004; Abdelwahab et al., 2013). Dexamethasone is also known to attenuate hyperalgesia by decreasing transmission of impulses in C- fibers (Sharififar et al., 2009). Thus, the extracts may have acted via a similar mechanism.

Daily administration of the ethyl acetate and aqueous fractions of the ethanolic extract and dexamethasone for 6 days attenuated the inflammation induced in the assay with dexamethasone completely reversing the inflammation within three days. Dexamethasone is known to inhibit leukocyte infiltration at the site of inflammation resulting in decreased release of bradykinin, TNF-α, IL-1, IL-2 and IL-6 (Zhang et al., 2014). Its action is also thought to involve phospholipase A_2 inhibitory proteins, lipocortins, which control the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotriene (Tsurufuji et al., 1984; Huang et al., 2013). Thus, like dexamethasone, the anti-inflammatory activity of the extracts may be as a result of decrease in the production of these mediators. This assertion was investigated by measuring serum levels of TNF-α, IL-1, IL-6 and IL-10 in the current assay at termination of treatment.

IL-1α, which activates the innate and adaptive immune responses, stimulates the production of INFγ by T lymphocytes (Arend, 2002; Chun et al., 2016). INFγ can induce IL-1α expression and enhance the cytotoxic action of TNF-α (Banno et al., 2004; Chun et al., 2016). TNF-α is an important cytokine involved in systemic inflammation, such as the one induced via adjuvant and acute phase response (Billiau, 1996; Jubri et al., 2013). It is released by white blood cells, endothelium and several other tissues in the course of damage and its release is stimulated by several mediators including IL-1α (Locksley et al., 2001).

IL-6 is a pro-inflammatory cytokine secreted by T cells and macrophages to stimulate the immune response to trauma and other tissue damage leading to inflammation (Hennes et al., 1996; Liu et al., 2014). Deregulation of IL-6 expression results in the synthesis and release of many inflammatory mediators which cause pain and edema, and it is a main factor for nociceptor excitation (Al-Hindawi et al., 1989; Yoshimura et al., 2009; Zhang et al., 2014). It has been shown that stimulation of IL-6 receptors on the afferent fibers of nociceptors can cause hyperalgesia during inflammation.

Elevation in serum levels of IL-6 increases the amount of secretion of some neurotransmitters such as substance P and calcitonin gene-related peptide through making an effect on β subunit of its receptors in nociceptors (Ruzek et al., 1997; Herder et al., 2013). The extracts of *C. sieberiana* decreased serum IL-6 as well as TNF-α and IL-1α which was aligned with hyperalgesia and edema reduction during the period of the study suggesting that the anti-inflammatory activity of the extracts may be due to their immunosuppressive effects via reduction in the synthesis of these pro-inflammatory cytokines.

IL-10, also known as human cytokine synthesis inhibitory factor, is an anti-inflammatory cytokine. Previous studies have suggested the function of IL-10 as an essential immunoregulator in the intestinal tract and, indeed, patients with Crohn's disease react favorably towards treatment with recombinant interleukin-10 - producing bacteria, demonstrating the importance of IL-10 for counteracting the hyperactive immune response (Braat et al., 2006). IL-10 is capable of inhibiting synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-3, TNF-α and GM-CSF made by cells such as macrophages and regulatory T-cells (Chun et al., 2016). The extracts elevated serum IL-10 confirming their immunoregulatory effects.

In all the assays carried out in this study, the ethyl acetate fraction had a slightly higher activity save in the TNF-α but this is not statistically significant. In our earlier study, we showed that both fractions have the same phytochemical constituents, namely saponins, flavonoids, anthraquinones and phenolic compounds (Donkor et al., 2013). Thus, it is not surprising that there were no significant difference in the activity of both fraction, and it is possible that these secondary metabolites found in the fractions may be implicated in the immunoregulatory activity of the extract.

Conclusion

Intraplantar injection of a mixture of low concentrations of heat killed *M. tuberclosis* and carrageenan successfully induce arthritis in the rat which is reversible via pharmacological intervention but not naturally. The anti-inflammatory activity of extracts of root bark of *C. sieberiana* may be attributable to their immunoregulatory effects via suppression of pro-inflammatory cytokines, TNF-α, IL-1α and IL-6; and elevation of the anti-inflammatory cytokine, IL-10 levels in serum.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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