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Antibacterial Activity of Some Compounds Isolated from *Aristolochia brevipes* and One Derivative of 9-methoxytariacuripyrone, Against Multiresistant Methicillin-Susceptible *Staphylococcus aureus* (MR-MSSA)

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ABSTRACT

Over the past two decades, Methicillin-resistant *Staphylococcus aureus* (MRSA) has evolved from a controllable infectious disease into a serious public health concern. MRSA is largely a hospital-acquired infection and is, in fact, one of the most common. Recently, however, new strains have emerged in communities that are capable of causing severe infection in otherwise healthy persons. The antibacterial activity of five major compound is evaluate from *Aristolochia brevipes* (dichloromethane extract) as follows: (1) 9-methoxytariacuripyrone, (2) 7,9-dimethoxytariacuripyrone, (3) E/Z-N-formylnornantenine, (4) 6α-7-dehydro-N-formylnornantenine, (5) aristololactam I, (6) an amino compound which obtained by means of biotransformation (with Baker's yeast), against Methicillin-sensitive *Staphylococcus aureus* (MSSA) and Methicillin-resistant *S. aureus* (MRSA). The structures of these compounds were elucidated by ^{1}H- and ^{13}C- (one-dimension [1D] and two-dimension [2D]) Nuclear Magnetic Resonance (NMR) spectroscopy. The 9-methoxytariacuripyrone compounds and the compound obtained by biotransformation, 5-amino-9-methoxy-3,4-dihydro-2H-benzo(h)chromen-2-one, showed highest activity against MRSA strains at a concentration range of 4.0-16.0 μg mL^{-1}. Hence, these compounds to hold promise as antibacterial agents in the treatment of infections with *S. aureus*, including MRSA and should be investigated further.

Key words: Antibacterial activity, 9-methoxytariacuripyrone, *Aristolochia brevipes*, methicillin-resistant *Staphylococcus aureus*

INTRODUCTION

The antibacterial properties of plant-derived compounds have been attracting attention, mainly in view of increased antibiotic resistance both in hospitals and in community-acquired infections (Moellering, 2006; Gould, 2008).

Identification of new compounds with antimicrobial effects has gained understandable interest, together with several recent reports of natural products on increasing the activity of antibiotics through synergistic interactions (D'Arrigo *et al*., 2010; Eumkeb *et al*., 2010).

Staphylococcus aureus is a Gram-positive, coagulase-positive coccus that occurs worldwide as part of the normal flora of animals and humans. There are over 30 *Staphylococcus* species, among which *S. aureus* is the most pathogenic of these for humans. In humans and animals,

S. aureus is a commensal colonizer of the skin and can also be found on mucous membranes of the body, particularly in throat, axilla, rectum and perineum as well as in the gastrointestinal tract. *Staphylococcus aureus* and Methicillin-resistant *S. aureus* (MRSA) are among the most significant Gram-positive pathogens. Because several MRSA strains have become multidrug-resistant endemic pathogens, novel therapies are needed to treat these widespread infections. Therefore, the use of natural antimicrobials could play a positive role in reducing infection rates (Moellering, 2012).

In this present study, the antibacterical activity of some constituents of the Mexican plant *Aristolochia brevipes*, is studied popularly known as "guaco", a plant that grows in several states of the Mexican Republic, such as Michoacán, Colima, Guerrero and Morelos and among others. Persons mainly use the rhizomes of this plant to treat arthritis, diarrhea, to cleanse wounds, for cough with blood and also to cure serpent wounds (Martinez, 1991; Achenbach *et al*., 1992, 1995). A dichloromethane extract of *A. brevipes* is prepared, from which the following five major compounds were isolated, (1) 9-methoxytariacuripyrone, (2) 7,9-dimethoxytariacuripyrone, (3) E/Z-N-formylnornantenine, (4) 6α-7-dehydro-N-formylnornantenine, (5) aristololactam I, previously isolated from *A. brevipes* (Achenbach *et al*., 1992, 1995) and (6) an amino compound, 5-amino-9-methoxy-3,4-dihydro-2H-benzo(h)chromen-2-one, obtained by biotransformation of the compound (1). These processes was carried out with the yeast *Saccharomyces cerevisiae* under culture (Alvarez-Fitz *et al*., 2012). This work reported the antibacterial activity of the previously mentioned compounds against one sensitive strain (Methicillin-sensitive *S. aureus*, MSSA) and four resistant strains (Methicillin-resistant *S. aureus*, MRSA), with important results. There is little information regarding the pharmacological activity of these compounds and in particular, antibacterial compounds. Therefore, according to opinion and knowledge, this is one of the first published reports.

MATERIALS AND METHODS

Collection of plant material: The root of *A. brevipes* was collected from its natural habitat in the municipality of Xochitepec in the Mexican state of Morelos during the month of September 2012. Herbolarium specimens were prepared which were deposited for classification at the Cuernavaca City, Morelos, National Institute of Anthropology and History Herbolarium by Margarita Aviles and Macrina Fuentes (INAHM-2033).

General procedure and equipment utilized: The compounds were purified by means of Open Column Chromatography (OCC) on Merck Kieselgel 60 gel. The isolation procedure and the purity of the compounds obtained were monitored by Thin Layer Chromatography (TLC) (Silica gel 60 F254 Merck, Germany), visualized by Ultraviolet light (UV) and revealed with ceric ammonium sulfate (Sigma Chemical Co., St. Louis, MO, USA). Infrared (IR) spectra were obtained in $CHCl_3$ on a Bruker Vector 22 infrared instrument. Nuclear Magnetic Resonance (NMR) experiments (^1H and ^{13}C) were obtained at 400 MHz for ^1H and at 100 MHz for ^{13}C in $CDCl_3$ on Varian Unity 400 equipment, utilizing Tetramethylsilane (TMS) as internal reference.

Extract preparation, separation and isolation of secondary metabolites: The extract of the dry ground root of *A. brevipes* (0.65 kg) was obtained by maceration with dichloromethane (1.5 L, 24 h×3). The macerated material was filtered and evaporated under low pressure in a rotatory evaporator. The extract obtained in this manner (9.5 g) was submitted to successive chromatographic processes by means of OCC. This procedure was initiated with 100% n-hexane as eluent system and polarity was gradually increased by means of successive additions of acetone to obtain the following five compounds, (1) 9-methoxytariacuripyrone, (2) 7,9-dimethoxytariacuripyrone, (3) E/Z-N-formylnornantenine, (4) 6α-7-dehydro-N-formylnornantenine, (5) aristololactam I. The structures of all of the compounds obtained were established unequivocally through analysis of their spectroscopic data of ^1H- and ^{13}C-NMR and by comparison of these with those published in the literature (Achenbach *et al*., 1992, 1995; Tantisewie *et al*., 1989; Che *et al*., 1984).

Biotransformation of 9-methoxytariacuripyrone with Baker's yeast: The process of biotransformation of 9-methoxytariacuripyrone by *Saccharomyces cerevisiae* yeast to obtain 5-amino-9-methoxy-3,4-dihydro-2H-benzo(h)chromen-2-one was performed as reported by Takeshita *et al*. (1989), modified by Alvarez-Fitz *et al*. (2012). In 500 mL Erlenmeyer flasks, distilled water (200 mL) is added and *Saccharomyces cerevisiae* yeast (50 g, NEVADA®, Edo. Mexico, Mexico), which were shaken at 160 rpm for 1-2 h and at a temperature of 30°C, the initial pH was 5.5. Subsequently, added the substrate 9-methoxytariacuripyrone (1.200 mg) dissolved in ethanol (2 mL) to the reaction flasks, while the control flasks only contained *Saccharomyces cerevisiae*. The stirring process continued for an additional 48 h under the same conditions. The transformation reaction was monitored using Thin Layer Chromatography (TLC), in which 10-15 mL aliquots were taken from the reaction mixture, extracted by partition with ethyl acetate (25 mL) and the organic phase was concentrated under reduced pressure for analysis utilizing 9-methoxytariacuripyrone (1) as reference. The mixture (pH was adjusted to 7.0 using 2 M sodium hydroxide solution) was extracted with ethyl acetate (500 mL×3 times). The organic phase was filtered through a Celite® bed, thereafter, the organic phase was concentrated at reduced pressure to obtain the extract that contains the reaction products. For isolation of the products, employed Open Column Chromatography

(OCC), for which silica gel is utilized (Merck Kiesegel 60, Darmstadt, Germany). This was eluted with a 9:1 hexane-acetone system, gradually increasing the polarity with acetone. The fractions and/or products obtained were monitored using TLC, observed with Ultraviolet (UV) light and revealed with ammonium sulfate solution (Sigma Chemical Co., St. Louis, MO, USA), the fractions were rechromatographed by means of OCC in order to obtain the pure products.

Microorganisms: The microorganisms used in this study included the following: *Staphylococcus aureus* (sensitive) American Type Culture Collection (ATCC) 6538 (MSSA), Staphylococcus aureus (β-lactamase producer) ATCC 11632 (MRSA1), *Staphylococcus aureus* (Methicillin-resistant) ATCC 33591 (MRSA2), *Staphylococcus aureus* (Methicillin-resistant) ATCC 43300 (MRSA3) and isolated clinically resistant Methicillin (MRSA4) which was obtained from a regional hospital of Mexican Institute of Social Security (IMSS) of Cuernavaca, Morelos, Mexico. The cultures were maintained in Mueller-Hinton (MH) agar (Merck, AMH) at 4°C until immediately prior to their use. The bacteria were inoculated in MH broth (Merck) at 37°C, 18 h prior to initiation of the test.

Determination of antibacterial activity: For antibacterial evaluation of the compounds, used an *S. aureus* strain and Methicillin-resistant *S. aureus* (MRSA). The compound from *A. brevipes* and the standard antibiotic (Chloramphenicol, Oxacillin and Ampicillin) were serially diluted in microplate wells at a concentration range of 0.5-128 μg mL^{-1}. The serial liquid microdilution method is utilized as described by Eloff (1998). The cultures were adjusted to 10^6 Colony-forming unit (CFU mL^{-1}) inoculum size, employing the standard 0.5 MacFarland scale. Five microliter of culture was added to each well which contained culture medium and the diluted samples. The microplates were placed under incubation at 37°C for 24 h. After this incubation period, 20 μL of p-iodonitrotetrazolium violet (0.5 mg mL^{-1}) was added to each microplate well. The formation of a red color is indicative of cellular viability. The Minimal Inhibitory Concentration (MIC) value was determined as the lowest concentration of the sample assayed that did not form the red color in the microplate well.

RESULTS AND DISCUSSION

The structures of all of the compounds obtained were established unequivocally through analysis of their spectroscopic data of ^{1}H- and ^{13}C-Nuclear Magnetic Resonance (NMR) and by comparison of these with those published in the literature (Achenbach *et al*., 1992, 1995; Tantisewie *et al*., 1989; Che *et al*., 1984) (Fig. 1a-f).

With respect to antimicrobial activity, all tested compounds showed inhibitory activity against the sensitive and resistant strains tested (Table 1), except for the alkaloid (3) which was not active. Compounds (1) and (6) were the most active compounds exhibiting an MIC value of 4-16 μg mL^{-1}, compound (2) which is different from compound (1) due to the presence of a second methoxy group, presumably exhibits decreased activity due to the presence of this other methoxyl (MIC, of between 32 and 64 μg mL^{-1}). Finally, the alkaloid (4) and aristolactam I, (5) demonstrated moderate antibacterial activity (Table 1).

Compounds (1) and (6) are phenolic-type compounds with a pyrone ring which could explain the antibacterial activity.

Many phenolic-type compounds with a pyrone ring (heterocyclic ring with oxygen and the carbonyl α, β unsaturated group; 2-pyrone) and pyrane (heterocyclic ring with oxygen and the carbonyl α, β unsaturated but in the position of heterocyclic 1, 4 with respect to a carbonyl group), such as coumarins, xanthones and flavonoid-type flavones, present antibacterial activity. In the literature, it has been found that coumarins and xanthones isolated from *Calophyllum brasiliense* and *Mammea americana* are the compounds responsible for activity against *S. aureus* (MSSA and MRSA); coumarins Mammea A/BA and A/AA and the xanthones Jacareubin and 1,3,5,6-Tetrahydroxy-2-(3,3-dimethylallyl), exhibiting an MIC between 1 and 8 μg mL^{-1} against all resistant bacteria tested (Yasunaka *et al*., 2005). Moreover, *Scutellaria barbata*-isolated apigenin and luteolin were active against MRSA (MIC, 3.9-15.6 μg mL^{-1}, for apigenin and 62.5-125 μg mL^{-1} for luteolin), these findings indicate that these compounds were uniformly active against all MRSA and MSSA strains (Sato *et al*., 2000).

Additionally, compounds containing nitro groups in their structure (nitroaromatic compounds) very rarely occur in nature (Gill *et al*., 1987), are widely used as antimicrobial agents against Gram-positive and -negative bacteria, protozoa and the occasional helminth and also exhibit selective toxicity

Table 1: Minimal Inhibitory Concentration (MIC) values of compounds and standard antibiotics against the sensitive strain and the four Methicillin-resistant *Staphylococcus aureus** (MRSA) strains

Compounds and antibiotics	MSSA	MRSA1	MRSA2	MRSA3	MRSA4
MIC (μg mL^{-1})					
1**	4.0	4.0	4.0	8.0	8.0
2	32.0	32.0	64.0	64.0	64.0
3	>128	>128	128	>128	>128
4	128	128	128	128	128
5	64	64	64	64	64
6	8.0	16.0	16.0	16.0	16.0
Chloramphenicol	0.5	2.0	2.0	2.0	2.0
Oxacillin*	2.0	32	64	128	128
Ampicillin*	2.0	64	128	128	128

*MIC: Minimal inhibitory concentration values of Oxacillin and Ampicillin = 4 μL were considered resistant strains, **1: 9-methoxytariacuripyrone, 2: 7,9-dimethoxytariacuripyrone, 3: E/Z-N-formylnornantenine, 4: 6α-7-dehydro-N-formylnornantenine, 5: Aristololactam I and 6: An amino compound derived from compound 1

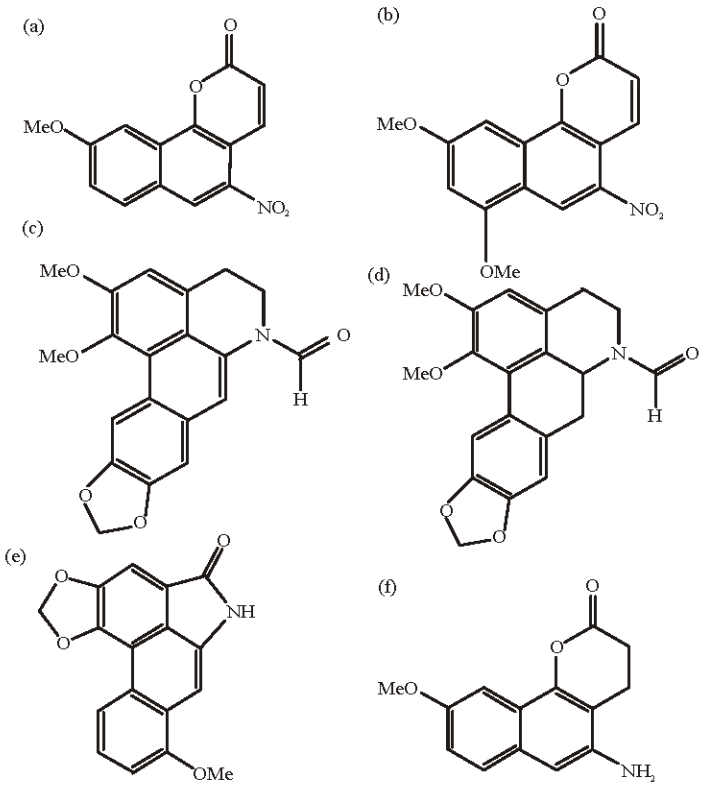

Fig. 1(a-f): Structures of the compounds from *Aristolochia brevipes*, (a) 9 methoxytariacuripyrone, (b) 7,9-dimethoxytariacuripyrone, (c) E/Z-N-formylnornantenine, (d) 6α-7-dehydro-N-formylnornantenine, (e) Aristololactam I and (f) Amino compound, 5 amino-9-methoxy-3-4, dihdro-2H-benzo(h) chromen-2 one

against anaerobic microorganisms. The mode of action of nitroaromatic compounds requires the reduction of the nitro group, involving the formation of nitroso and hydroxylamine intermediates; the biological target is the DNA and reducing the intermediates causes of strand breakage and helix destabilization, biologically inducing damage (Tocher, 1997), which could explain the antibacterial activity of 9-methoxytariacuripyrone.

It is noteworthy that there are no reports in the literature on the antibacterial activity against MRSA of these compounds isolated from *A. brevipes*. Thus, the present knowledge, this would be the first report of the biological activity.

The present study supports the fact that the organic extract of *A. brevipes* provides an excellent opportunity to find active molecules. Two compounds, 9-methoxytariacuripyrone and the compound obtained by biotransformation, exhibited important activity against four methicillin-resistant *S. aureus* strains.

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