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ANTILEISHMANIAL EPIDIOXYSTEROLS FROM THE COLOMBIAN MARINE SPONGE *Ircinia campana* ARE OXIDATION PRODUCTS FROM NATURALLY OCCURRING $\Delta^{5,7}$ STEROLS

LOS EPIDIOXIESTEROLES CON ACTIVIDAD ANTILEISHMANIA DE LA ESPONJA MARINA COLOMBIANA *Ircinia campana* SON PRODUCTOS DE OXIDACIÓN DE LOS ESTEROLES NATURALES $\Delta^{5,7}$

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ABSTRACT

Marine sponges of the genus *Ircinia* are known to contain several classes of metabolites, some of them with interesting biological activities as furanesesterterpenes, polyprenylated hydroquinones, macrolides, sulfur containing terpenoids, and steroids with various patterns of oxidation. In our search for antiparasitic metabolites from marine sponges, we found that the Colombian Caribbean sponge *Ircinia campana* contains a complex mixture of epidioxysterols which displayed antileishmanial activity. This paper demonstrates that epidioxysterols found in this sponge are photo-oxidation products from the naturally occurring $\Delta^{5,7}$ sterols. These results suggest that epidioxysterols reported previously in sponges of the genus *Ircinia* are not naturally occurring products, so they are not valid chemotaxonomic markers for the sponges of this genus.
Keywords: Marine sponges, Dyctioceratidae, *Ircinia*, epidioxysterols, degradation products.

RESUMEN

Las esponjas marinas del género *Ircinia* contienen varias clases de metabolitos, algunos de ellos con actividades biológicas interesantes como son: furanosesterterpenos, hidroquinonas polipreniladas, macrólidos, terpenoides azufrados, y esteroides con diferentes patrones de oxidación. En nuestra búsqueda de sustancias antiparasitarias en esponjas marinas, encontramos que la esponja marina colombiana *Ircinia* campana contiene una mezcla compleja de epidioxiesteroles, los cuales mostraron actividad antileishmania. Este trabajo demuestra que los epidioxiesteroles en esta esponja, son productos de la oxidación fotoquímica de sus esteroles naturales $\Delta^{5,7}$. Los resultados que se presentan sugieren que los epidioxiesteroles reportados previamente en otras esponjas del género *Ircinia* no son productos naturales y, por tanto, no son marcadores quimotaxonómicos válidos para este género de esponjas.
Palabras clave: Esponjas marinas, Dyctioceratidae, *Ircinia*, epidioxiesteroles, productos de degradación.

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INTRODUCTION

Sponges are sessile animals that produce bioactive substances as a mechanism of defense against their predators. This ability has been confirmed with the finding of a lot of new substances, most of them with structural and biological properties without counterpart in the terrestrial environment. In the particular case of marine sponges of the genus *Ircinia*, these are known as a source of several classes of steroids (1, 2), terpenoids (3, 4, 5, 6), furanoseterterpene tetronic acids (6, 7, 8), hydroquinones polyprenylated (9), sulfur compounds as ircinia-sulfonic acid (10, 11), nytrogenous compounds (12) and macrolides (13). Several of these compounds displayed interesting biological activities including enzyme inhibition (4), antitumoral)(12-13), and antimicrobial activities(14).

In a previous study we found that extracts from the Colombian Caribbean marine sponge *Ircinia campana*, displayed interesting antiparasitic activity which was associated with a complex mixture of epidioxysterols isolated from the sponge (15). In this article we report that these compounds are products of oxidation from the naturally occurring $\Delta^{5,7}$ sterols in the presence of light.

Sterols $\Delta^{5,7}$-3-hydroxyandrostadiene were obtained using a bioassay guided fractionation from the Colombian marine sponge *Ircinia campana*. The sterol fraction was oxidated in controlled conditions of light and air to produce 5α,8α-epidioxysterols which were identified by GC/MS. Nine 5α, 8α-epidioxysterols were identified: Six compounds with Δ^6 nucleus and three compounds with $\Delta^{6,9}$ (11) nucleus. This fraction showed antileishmanial activity against *Leishmania (V.) panamensis*.

MATERIALS AND METHODS

General experimental procedures

All solvents were of the highest commercially available purity. UV and FTIR spectra were recorded on a Genesis 2PC spectrophotometer and a Perkin Elmer (FTIR Spectrum I) spectrophotometer, respectively. ^{1}H-NMR measurements in $CDCl_3$ were performed on a Bruker AMX 300 instrument, operating at 300 MHz with TMS as an internal reference. GC/MS: Agilent GC 6890/ MS 5973, with a capillary column HP5-MS (30 m x 0,25 mm x 0,25 μm film). Carrier gas: He 0.9 ml min^{-1}, temperature program: 200 °C – 290 °C at 5 °C min^{-1}. An Agilent 1100 system with ultraviolet detection (monitored at 210 nm) was used for the analysis of fractions. A Merck LiChrospher 100 RP-18 reversed phase column (10 μm x 250 mm) were all eluted isocratically with acetonitrile: methanol: water (8:1:1) at a flow rate of 1mL/min. Analytical TLC was performed on precoated Merck aluminum sheets (DC-Alufolien Kieselgel 60 F_{254}, 0.2 mm) with the solvent system $CHCl_3$-methanol (95:5, 80:20 and 60:40), and compounds were viewed under UV lamp and sprayed with 5% phosphomolibdic acid in ethanol followed by heating. Column chromatography analysis were performed using silica gel 40.

Animal material

Samples of sponge *Ircinia campana* were collected and identified during November of 2001 and March of 2002 in Punta Betín, Santa Marta at 6-12 m depth. Voucher specimen with code INV-POR 0022 has been deposited in the collection sponges of INVEMAR, Santa Marta (correspond to the dried fragment of the whole specimen deposited in the Smithsonian Institution in Washington with code USNM 32003). Samples of *Ircinia campana* were immediately frozen after collection and kept at 4 °C until extraction.

Extraction and fractionation

Frozen sponges were cut into small pieces, lyophilized and ground. Milled sponges (66.33 g and 255.94 g) were extracted with methanol at room temperature. The methanolic extracts were dried at 40 °C on a rotatory evaporator. The extracts were re-extracted with ethyl acetate and the sterol fraction (374.9 mg and 1526.3 mg) were separated and purified by TLC and CC (silicagel F_{254} and silicagel 40, respectively, *n*-hexane: ethyl acetate 2:1).

Oxidation of $\Delta^{5,7}$ sterols to epidioxysterols

$\Delta^{5,7}$ sterols were dissolved in chloroform and kept in a open reflux with direct light of a halogen lamp (50 Watts, 120 Volts), Philips® Master N-Flood 30, and constant agitation for 24 hours at room temperature. The reaction control was monitored using the same conditions for TLC and compared with a control sample without oxidize and with a commercial sample of 7-dehydrocholesterol. On TLC analysis (silica gel, n-hexane/ethyl acetate 2:1) sterols with $\Delta^{5,7}$-3β-hydroxyandrostadiene nuclei displayed Rf 0.6 and showed an UV absorption at 254 nm, while epidioxysterols displayed Rf 0.2 and it cannot be revealed with UV light owing to ab-

sence of conjugated diene system. The sample was fractionated by CC and eluted with the eluent series chloroform-methanol (95:5, 80:20 and 60:40). Two fractions (A and B) were obtained, and analyzed by ^{1}H-NMR, HPLC and GC/MS. GC/MS analysis of fraction A led to identification of 6 compounds: 5α,8α-epidioxy-24-norcholesta-6,22-dien-3β-ol (1), 5α,8α-epidioxy-cholesta-6,22-dien-3β-ol (2), 5α, 8α-epidioxi-(24)-methylcholest-6-en-3β-ol (epimer 1) (3), 5α, 8α-epidioxy-cholest-6-en-3β-ol (4), 5α, 8α-epidioxy-(24)-methylcholest-6-en-3β-ol (epimer 2) (5), 5α, 8α-epidioxy-(24)-ethylcholesta-6,22-dien-3β-ol (6), and GC/MS analysis for fraction B led to identification of 3 compounds: 5α,8α-epidioxy-cholesta-6,9-dien-3β-ol (7), 5α,8α-epidioxy-cholesta-6,9,22-trien-3β-ol (8), and a compound 5α,8α-epidioxysterol with molecular formula $C_{27}H_{42}O_4$ (9).

RESULTS

Two extraction and isolation processes to find the epidioxysterols fraction from *Ircinia campana* were unsuccessful. In the first case, isolation work began with 66.33 g of dried sponge, and in the second case it was increased to 255.9 g of this specie. All processes for extraction were developed protecting from light, using amber glass recipients. No evidence of the presence of epidioxysterols in crude extracts was available. However, in both cases the sterol fractions were isolated. Evidence for the presence of sterols with a $\Delta^{5,7}$ nuclei were found from TLC and UV analysis, and from previous reports of these class of sterols in sponges genus *Ircinia* (16). Sterol fractions were isolated by repetitive column chromatography and the UV spectrum displayed characteristic UV maximum for sterols with a $\Delta^{5,7}$ nuclei at 280 nm.

Considering the previous suggestion made by Martinez in 1996, who reported the sterol composition of three *Ircinia* species from the Caribbean sea, and a small quantity of epidioxysterols in *Ircinia campana*, which could be oxidation products from the corresponding naturally ocurring $\Delta^{5,7}$ sterols in the sponge, we were encouraged to test the oxidation of sterol fractions isolated from *Ircinia campana*.

In a process of analysis and fractionation by thin layer chromatography and column chromatography, the fraction of epidioxysterols did not present the retention factor of 0.23 when it was eluted with a mixture of n-hexane/ ethyl acetate 2:1. Moreover, when ^{1}H-NMR spectrum (300 MHz, $CDCl_3$) was determinated, characteristic signals of olefinic protons H-6 and H-7 of epidioxysterols were not observed (14), neither for lipidic extract nor the fractions achieved by chromatographyc fractionation.

After the oxidation of $\Delta^{5,7}$ sterols, the analysis of ^{1}H-NMR spectrum of a purified fraction of oxygenated sterols, showed characteristic signals of methyl protons between δ 0.6 and 1.0, a multiplet at δ 3.9 that correspond to proton H-3, a doublet at δ 6.24 (*d*, J 8,5 Hz) that correspond to olefinic proton H-7 and a doublet signal at δ 6.50 (d, J 8.5 Hz) correspond to olefinic proton H-6. This fact confirmed that the fraction was a mixture of compounds with the characteristic nucleus of epidioxyserols, of which nine compounds were identified by GC/MS: 5α,8α-epidioxy-24-norcholesta-6,22-dien-3β-ol (1), 5α,8α-epidioxy-cholesta-6,22-dien-3β-ol (2), 5α,8α-epidioxi-(24)-methylcholest-6-en-3β-ol (epimer 1) (3), 5α,8α-epidioxy-cholest-6-en-3β-ol (4), 5α,8α-epidioxy-(24)-methylcholest-6-en-3β-ol (epimer 2) (5), 5α,8α-epidioxy-(24)-ethylcholesta-6,22-dien-3β-ol (6),5α,8α-epidioxy-cholesta-6,9-dien-3β-ol (7), 5α,8α-epidioxy-cholesta-6,9,22-trien-3β-ol (8), and a 5α,8α-epidioxysterol with molecular formula $C_{27}H_{42}O_4$ (Figure 1).

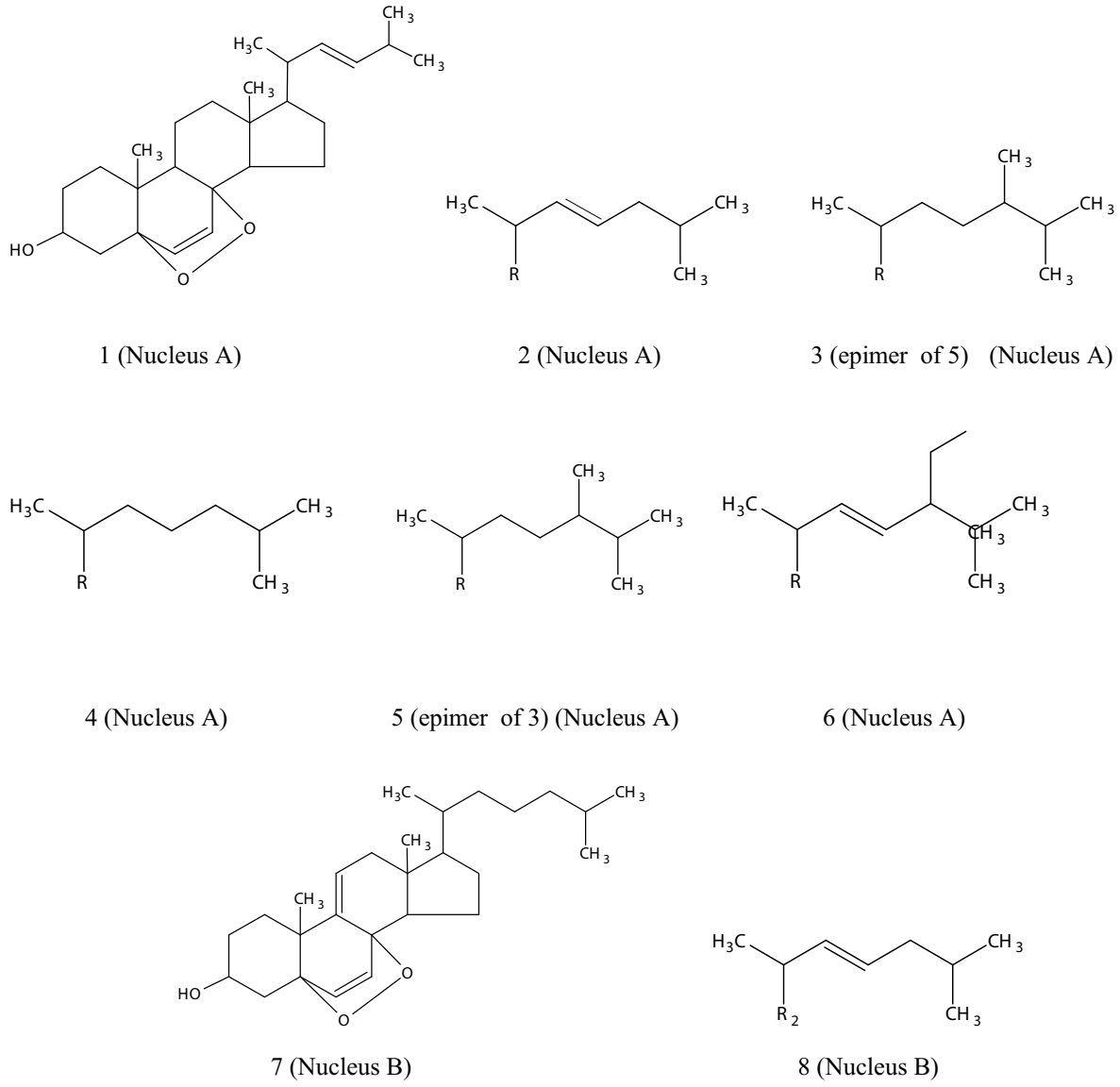

Figure 1. Structures of 5α,8α-epidioxysterols from the marine sponge *Ircinia campana.*

DISCUSSION

Previous results suggested that the epidioxysterols in the marine sponge *Ircinia campana* are artifacts of oxidation of sterols with $\Delta^{5,7}$-3β-hydroxyandrostadiene nucleus. This fact had been suggested by Martinez in 1996, which explained the oxidation reaction for this compounds (Figure 2). In addition, results suggest that the epidiosxysterols reported for marine sponges *Ircinia campana* and *Ircinia fasciculata* (17), were not naturally ocurring products in these sponges, but may be the artifacts of oxidation in presence of light. This hypothesis reasserted from the fact that in both processes of extraction, were carried out in the absence of light (in ambar glass container) from their collection to isolation of sterols fraction.

In a revision of scientific literature about the oxidation process of sterols with $\Delta^{5,7}$-3β-hydroxyandrostadiene nucleus, it was reported that 5α,8α-epidioxysterols present in the sponge *Spheciospongia vesparia*, are artifacts of oxidation of natural $\Delta^{5,7}$-sterols. However, in that study researchers produced the oxidation with an oxidant agent, *m*-chlorperbenzoic acid. (18). These results suggest that the epidioxysterols found in marine sponges cannot be used as chemotaxonomic markers because they are probably oxidation artifacts.

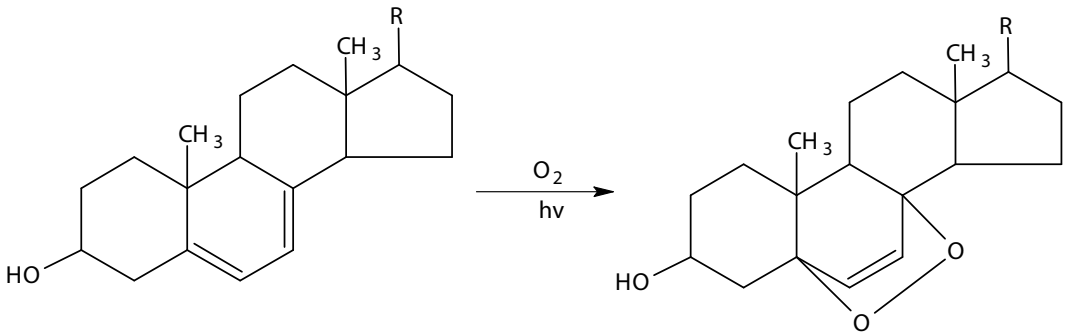

Figure 2. Oxidation reaction of $\Delta^{5,7}$- natural sterols from *Ircinia campana*.

Spectroscopic Data

Epidioxysterols mixture: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0,6-1,0 (15H, m, CH_3-18, CH_3-19, CH_3-21, CH_3-25, CH_3-26), δ 3,90 (1H, m, H-3), δ 6,24 (1H, d, J = 8,5 Hz, H-7), δ 6,50 (1H, d, J = 8,5 Hz, H-6).

5 α,8 α-epidioxy-24-norcholesta-6,22-dien-3β-ol (1): EIMS *m/z* 400 $[M]^+_{\cdot}$, 368, 353, 349. Retention time by GC, 15.08 min. These data are consistent with a epidioxysterol of molecular formula $C_{26}H_{40}O_3$.

5α,8α-epidioxy-cholesta-6,22-dien-3β-ol (2): EIMS *m/z* 414 $[M]^+_{\cdot}$, 382, 396, 364, 349. Retention time by GC, 16.21 min. These data are consistent with a epidioxysterol of molecular formula $C_{27}H_{42}O_3$.

5α,8α-epidioxy-(24)-metilcholest-6-en-3β-ol (3) – Epimer 1: EIMS *m/z* 430 $[M]^+_{\cdot}$ 412, 398, 380, 365, 353. Retention time by GC, 17.90 min. These data are consistent with a epidioxysterol of molecular formula $C_{28}H_{46}O_3$.

5α,8α-epidioxy-cholest-6-en-3β-ol (4): EIMS *m/z* 416 $[M]^+_{\cdot}$, 412, 398, 366, 353, 351. Retention time by GC, 19.07 min. These data are consistent with a epidioxysterol of molecular formula $C_{27}H_{44}O_3$.

5α,8α-epidioxy-(24)-methylcholest-6-en-3β-ol (5) – Epimer 2: EIMS *m/z* 430 $[M]^+_{\cdot}$, 412, 398, 380, 365, 353. Retention time by GC, 19.40 min. These data are consistent with a epidioxysterol of molecular formula $C_{28}H_{46}O_3$.

5α,8α-epidioxy-(24)-etilcholesta-6,22-dien-3β-ol (6): EIMS *m/z* 442 $[M]^+_{\cdot}$, 424, 410, 392, 377, 253. Retention time by GC, 20.64 min. These data are consistent with a epidioxysterol of molecular formula $C_{29}H_{46}O_3$.

5α,8α-epidioxycholesta-6,9(11)-dien-3β-ol (7): EIMS *m/z* 414 $[M]^+_{\cdot}$, 396, 382, 364, 349, 353. Retention time by GC, 21.44 min. These data are consistent with a epidioxysterol of molecular formula $C_{27}H_{42}O_3$.

5α,8α-epidioxy-cholesta-6,9(11),22-trien-3β-ol (8): EIMS *m/z* 412 $[M]^+_{\cdot}$, 394, 380, 365, 253. Retention time by GC, 23.45 min. These data are consistent with a epidioxysterol of molecular formula $C_{27}H_{40}O_3$.

Compound 9:. EIMS *m/z* 430 $[M]^+_{\cdot}$, 412, 398, 365, 253. Retention time by GC, 21.79 min. These data are consistent with a epidioxysterol of molecular formula $C_{27}H_{42}O_4$.

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BIBLIOGRAPHIC REFERENCES

1. Kobayashi J, Shinonaga H, Shigemori H. Xestobergsterol C, A New Pentacyclic Steroid from the Okinawan Marine Sponge *Ircinia sp*. and Absolute Stereochemistry of Xestobergsterol A. J Nat Prod 1995; 58: 312-318.
2. Venkateswarlu Y, Reddy MVR, Rao MR. A New Epoxysterol from the Sponge *Ircinia fasciculata*. J Nat Prod 1996; 59:876-877.
3. Shen Y-C, Lo K-L, Lin Y-C, Khalil AT, Kuo Y-H,Shih P-S. Novel Linear C_{22}-Sesterterpenoids from Sponge *Ircinia formosana*. Tetrahedron Lett 2006; 47(24): 4007-4010.
4. Buchanan MS, Edser A, King G, Whitmore J, Quinn RJ. Cheilanthane Sesterterpenes, Protein Kinase Inhibitors, from a Marine Sponge of the Genus *Ircinia*. J Nat Prod 2001; 64: 300-303.
5. Wright AE, McCarthy PJ. Sulfircin: A New Sesterterpene Sulfate from a Deep-Water Sponge of the Genus *Ircinia*. J Org Chem 1989; 54: 3472-3474.
6. Höller U, König GM and Wright AD. Two New Sesterterpene Tetronic Acids from the Marine Sponge *Ircinia oros*. J Nat Prod 1997; 60: 832-835.
7. Martínez A, Duque C, Sato N, Fujimoto Y. (8Z,13Z,18R,20Z)-Strobilinin and (7Z,13Z,18R,20Z)-Felixinin: New Furanosesterterpene Tetronic Acids from Marine Sponges of the Genus *Ircinia*. Chem Pharm Bull 1997; 45: 181.
8. Barrow CJ, Blunt JW, Munro MHG, Perry NB. Oxygenated Furanosesterterpene Tetronic Acids from a Sponge of the Genus *Ircinia*. J Nat Prod 1988; 51: 1294-1298.
9. De Rosa S, Crispino A, De Giulio A, Iodice C, Milone A. Sulfated Polyprenylhidroquinones from the Sponge *Ircinia spinulosa*. J Nat Prod 1995; 58: 1450-1454.
10. Kawakami A, Miyamoto T, Higuchi R, Uchiumi T, Kuwano M, Van Soest RWM. Structure of a Novel Multidrug Resistance Modulator, Irciniasulfonic Acid, Isolated from a Marine Sponge, *Ircinia* sp. Tetrahedron Lett 2001; 42: 3335-3337.
11. Emura C, Higuchi R, Miyamoto T. Irciniasulfonic Acid B, a Novel Taurine Conjugated Fatty Acid Derivative from a Japanese Marine Sponge, *Ircinia* sp. Tetrahedron 2006; 62(24): 5682:5685.
12. Kondo K, Shigemori H, Kikuchi Y, Ishibashi M, Sasaki T, Kobayashi J. Ircinals A and B from the Okinawan Marine Sponge *Ircinia* sp.: Plausible Biogenetic Precursors of Manzamine Alkaloids. J Org Chem 1992; 57: 2480-2483.
13. Rashid MA, Cantrell ChL, Gustafson KR, Boyd MR. Chondropsin D, a New 37-Membered-Ring Macrolide Lactam from the Marine Sponge *Chondropsis* Species. J Nat Prod 2001; 64:1341-1344.
14. Salama AM, Gamboa M, Pinzón M. Actividad antimicrobiana y antitumoral de la variabilina y sus enantiómeros aislados de *Ircina felix*. Rev Col Cienc Quím Farm 2001; 30:74-80.
15. Martínez A, Robledo S, Muñoz D, Blair TS, Higuita DE, Echeverri PE*, et al.* Antiparasitic Activity of Methanol Extracts and Isolated Fractions from Caribbean Sponges. Vitae 2001; 8 (1 y 2): 71-81.
16. Martínez, A. Estudio químico y de actividad biológica de esponjas marinas colombianas del género *Ircinia*. Tesis doctoral. Universidad Nacional de Colombia., 1996.
17. Calderòn JS, Quijano L, Gómez F, Acosta F, Ríos T. Estudios preliminares sobre algunas esponjas del litoral mexicano. I. Rev Latinoam Quím 1982; 13: 49-52.
18. Arreguín-Espinosa R, Arreguín B, Hernández-Santoyo A, Rodríguez-Romero A. Sterol Composition and Biosynthesis in the Sponge *Spheciospongia vesparia*. J Chem Technol Biotechnol 1998; 72: 245-248.