Unraveling the anti-biofilm properties of Laurinterol on pioneer biofouling bacteria from the red seaweed Laurencia johnstonii

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Abstract

A biofilm is a complex microbial community, representing the initial stage in biofouling formation. Consequently, it is responsible for significant economic losses in several industrial sectors worldwide. Therefore, there is a constant need for safer and environmentally friendly coatings, particularly those derived from new marine sources such as seaweeds with antifouling properties. Red algae produce metabolites that prevent bacterial attachment and biofilm formation by disrupting microbial membranes, inhibiting quorum sensing, or interfering with extracellular matrix production, among them Laurencia johnstonii. This species has particular ecological or geographical advantages that make it more accessible or abundant for research in the Bay of La Paz, BCS. This study aimed to assess the anti-biofilm potential of the red seaweed Laurencia johnstonii. To identify bioactive compounds, the anti-biofilm activity of the ethanolic extract was evaluated against marine biofilm-forming strains: Bacillus altitudinis, Bacillus pumilus, Bacillus subtilis, and Bacillus cereus. The ethanolic extract of L. jonhstonii exhibited the highest percentage of inhibition. Subsequent chromatographic fractionation led to the isolation and identification of laurinterol, the primary compound responsible for the anti-biofilm activity (>97 %) and antibacterial activity (MIC <3.9 µg/mL). To our knowledge, this is the first report of the activity of laurinterol against biofilm-forming strains.

Introduction

Biofilms are complex microbial communities that attach to a substrate surface or an interphase. Furthermore, microbial biofilms can adhere to a living surface or tissues growing in a self-secreted extracellular matrix (Donlan 2002). In healthcare, biofilm formation complicates the treatment of infectious diseases and contributes to bacterial resistance to common antibiotics, the main cause of persistent infections (Zhao et al. 2023). In marine environments, microbial biofilms are present on a wide range of artificial objects, from bottles to oil platforms, resulting in significant economic losses in several sectors worldwide (Qian et al. 2022). Moreover, the formation of this matrix facilitates microbial aggregation, a crucial step in the colonization of surfaces by microorganisms such as barnacles, mussels, and macroalgae among others. Biofilm represents the initial stage in the development of biofouling (Wang et al. 2022).

Marine fouling organisms cause a variety of damages beyond increased drag and reduced energy efficiency in ships and vessels (Schultz et al. 2010). Their attachment to the surfaces such as piers, docks, and underwater pipelines accelerates corrosion and wear. Additionally, the weight and abrasive nature of these organisms can compromise the structural integrity of boats, offshore oil rigs, and subsea infrastructure (Hopkins et al. 2021).

In aquaculture systems, fouling organisms compete for space and nutrients with farmed species, leading to reduced growth rates and increased stress. Furthermore, biofouling also facilitate the dissemination of pathogens and diseases within aquaculture systems. Organisms that settle on nets, cages, and tanks may harbor harmful microorganisms, which can infect the farmed species, resulting in

economic losses and potential environmental harm (Bannister et al. 2019). Antifouling coatings are a common solution to this problem; however, they often contain toxic compounds such as copper, other metals, or the broad-spectrum biocide TBT, which causes significant damage to the marine environment (Abioye et al. 2019). Therefore, novel environmentally friendly coatings derived from marine sources are being developed as safer alternatives.

Furthermore, marine biofilms contribute to ecological challenges due to their ability to colonize diverse biological surfaces, including plankton, algae, and animals. Seaweeds have evolved in the marine environment over millions of years, where biofouling is a constant threat. To counteract this, they have developed adaptive traits that inhibit microbial adhesion, reducing vulnerability to fouling and ensuring optimal access to sunlight and nutrients. This mechanism probably involves complex evolutionary pressures, including the need to remain free of microbial colonies that could obscure the algal surface or interfere with the photosynthetic process, growth, and distribution (Qian et al. 2022). As a result, some seaweeds have evolved effective chemical defenses against microbial epibionts, producing secondary metabolites with antibacterial and antifouling properties (Hellio & Yebra 2009; Zammuto et al. 2022).

Some of the compounds produced by algae are particularly effective against pioneer bacterial species—the first to colonize and form biofilms. By inhibiting the attachment of these initial settlers, algae reduce the risk of more complex biofilm formation, which could involve a broader range of microorganisms and potentially harmful organisms such as barnacles or other larger marine invertebrates. These metabolites interfere with bacterial attachment and biofilm formation by disrupting microbial cell membranes (Feng et al. 2022), inhibit quorum sensing (the signaling mechanism that bacteria use to coordinate biofilm formation) (Goecke et al 2010; Behzadnia et al. 2024), or affecting the production of extracellular matrix components that bind bacteria together (Rima et al. 2022; Behzadnia et al. 2024). These antimicrobial compounds act as a chemical defense, preventing microbial colonization and biofilm formation on the algal surface. These antifouling properties may offer an alternative to current methods and could help to reduce the toxic effects observed in the oceans. Previous reports on antifouling activity highlight the potential of red seaweeds (Da Gama et al. 2008; Aguila-Ramírez et al. 2012; Pinteus et al. 2021), particularly the genus *Laurencia*, which is considered one of the main sources of active metabolites with around 800 identified compounds, mainly terpenoids (Yamagishi et al. 2024). These compounds often demonstrate potent properties, making *Laurencia* an ideal candidate for biofilm inhibition and antimicrobial activity research.

Our previous research demonstrated the anti-biofilm potential of an ethanolic extract of *Laurencia johnstonii* (Aguila-Ramírez et al. 2012). These findings suggest *Laurencia* as a promising candidate for further investigation. Therefore, this study aims to evaluate the anti-biofilm activity of the red seaweed *Laurencia johnstonii* and identify the active compounds.

Materials and methods

Algal material and ethanolic extract preparation

Laurencia johnstonii was collected at Coyote Beach (24° 21' 09.2" N–110° 16' 23.5" W) in Bahia de La Paz, Mexico. Taxonomic identification was assessed by morphological characters (Abbott & Hollenberg 1976) and confirmed by Dr. Juan Manuel López Vivas at Marine Botany Laboratory (Universidad Autónoma de Baja California Sur, Mexico).

For the ethanolic extract, 100 g of air-dried seaweed were ground to 40-mesh size and macerated with 250 mL of distilled ethanol for 24 h. The ethanolic extract was concentrated using an RII rotavapor evaporator (Buchi, Switzerland) at 40°C. The extract was stored at -20°C until further analysis.

Fractionation of Laurencia johnstonii

For isolation, 3 g of *L. johnstonii* extract were separated by solid-liquid extraction using an elution gradient of *n*-hexane, dichloromethane, ethyl acetate, and methanol. This process yielded four fractions: F1, F2, F3, F4.

Fraction F2 (1 g) was further fractionated on a silica gel column (70–230 mesh) with an elution gradient of *n*-hexane, dichloromethane, and methanol, resulting in five fractions: F2C1, F2C2, F2C3, F2C4, F2C5. To isolate the anti-biofilm compounds, the active fraction F2C2 was further fractionated on a silica gel column (70–230 mesh) with an elution gradient of *n*-hexane, dichloromethane, and methanol, yielding two final fractions: F2C2A, F2C2B. The sesquiterpene laurinterol (8 mg) was isolated from the F2C2A fraction by high-performance liquid chromatography (HPLC) (Fig. 1). Preparative chromatography was conducted on an HPLC system consisting of a 2335 quaternary gradient module and a 2998 photodiode array detector (Waters Corporation). Sample was injected manually with a 500 µL loop. Separation was conducted on a C18 prep OBD column (5 µm, 10 x 250 mm, Waters), using a mobile phase consisted of 10 mM ammonium acetate buffer (solvent A) and acetonitrile (solvent B) with the following elution gradient: 0–5 min, 60% B; 40 min, 100% B; 50 min, 100% B, 55 min, 60% B. The column was operated at ambient temperature with a flow rate of 3.5 mL/min, and the run time was 55 min. UV detection was set at 280 nm.

Figure 1. Bio-guided fractionation of *Laurencia johnstonii* extract to obtain laurinterol.

Chemical characterization of laurinterol

NMR spectrum was recorded on a Bruker AVANCE 500 MHz by dissolving the sample in $CDCl_3$ (99.9%), with chemical shifts reported relative to solvent (7.26 ppm) and TMS as an internal pattern. The NMR data were compared with those previously reported in the literature to confirm the structure (García-Davis et al. 2019; González-Castro et al. 2024).

Laurinterol: $C_{15}H_{19}BrO$, ^1H NMR ($CDCl_3$) 0.54 (1H, t, J = 3.9, H-12), 0.57 (1H, m H-12), 1.14 (1H, dt, J = 8.1, 4.2 Hz, H-3), 1.28 (1H, d, J = 4.5 Hz, H-5), 1.40 (3H, s, H-13), 1.57 (3H, s, H-14), 1.66 (1H, dd, J = 12.4, 8.0

Hz, H-4), 1.94 (1H, tdd, J = 12.3, 8.2, 4.4, H-4), 2.08 (1H, dd, J = 13.2, 8.1 Hz, H-5), 2.29 (3H, s, H-15), 5.13 (1H, Br, s, 7-OH), 6,61 (1H, s, H-8), 7.60 (1H, s, H-11).

Figure 2. Laurinterol sesquiterpene isolated from *Laurencia johnstonii.*

Bacteria and culture media

Pioneer bacteria were isolated from acrylic and fiberglass plates submerged in the sea for 6 h. To efficiently select strains that could adhere and form biofilms, a rapid method based on the crystal violet staining of biofilms formed in 96-well microtiter plates was employed to identify bacterial strains with high biofilm-forming abilities. The bacterial strains were identified as *Bacillus altitudinis*, *B. pumilus*, *B. subtilis*, and *B. cereus* Bacterial cultures were grown on tripticasein soy agar with 2.7% NaCl (TSA-NaCl). Before the assay, the bacterial strains were suspended in saline solution and adjusted to 1×10^8 cells mL^{-1} (Merck SQ118).

Anti-biofilm activity assay

The *in vitro* anti-biofilm activity was assessed using the crystal violet assay in a 96-well flat-bottom plate. Each well was inoculated with 100 µL of bacterial suspension and 100 µL of extract (1 mg mL^{-1}). The plates were incubated at 35 ºC for 48 h (Heratherm Thermo Scientific). All assays were performed in triplicate, with a blank control (broth without bacteria) and a negative control (broth with bacterial suspension) included. After incubation, the wells were washed with distilled water to remove non-adherent cells, and the cells were fixed with 250 µL of methanol for 15 min. After removing the methanol, the plates were air-dried for 45 minutes. Next, 200 µL of 1% crystal violet solution was added, and the plates were left at room temperature for 20 minutes. The crystal violet was removed, and the plates were rinsed with distilled water until any excess dye was cleared and the plates were allowed to dry. Finally, 250 µL of ethanol (96%) was added to solubilize the crystal violet adhered to the plate. The biofilm mass was quantified by measuring the absorbance of the destaining solution at 595 nm using a microtiter plate reader (Infinite M1000 PRO, Tecan) (Shukla & Rao 2017).

The biofilm inhibition (%) was calculated using the following equation:

$$\left[1 - \left(\frac{A_{extract} - A_{blank}}{A_{control}}\right) x\ 100\right]$$

Where, $A_{control}$: negative control absorbance, A_{blank}: blank absorbance, $A_{extract}$: extract absorbance

Minimal inhibitory concentration (MIC) determination

The extract was serially diluted to create a concentration gradient ranging from 1000 µg mL^{-1} to 0.97 µg mL^{-1}. All samples were subjected to triplicate analysis, along with the blank (broth without bacteria) and

the negative control (broth with bacterial suspension), following the same methodology mentioned above.

Results

Anti-biofilm activity

The ethanolic extract exhibited strong activity against biofilm formation in most tested strains, and fraction F2C2 showed the highest inhibition of biofilm formation across all bacterial strains (Table 1). Consequently, this fraction was further fractionated, and the two resulting fractions, F2C2A, and F2C2B, were also evaluated. Both fractions showed strong inhibition of biofilm formation at a concentration of 0.06 mg·mL^{-1}.

Table 1
Percentage of inhibition of biofilm formation (%) of the ethanolic extract of *Laurencia johnstonii* and its fractions [1 mg mL^{-1}]. *Calculated at a concentration of [0.06 mg mL^{-1}], (n = 3).

	B. altitudinis	***B. pumilus***	***B. subtilis***	***B. cereus***
Ethanolic extract	79.40 ± 3.9	100 ± 3.3	100 ± 0.5	12.11 ± 0.8
F2C1	93.33 ± 3.7	61.47 ± 0.4	90.78 ± 2.8	87.15 ± 2.1
F2C2	94.24 ± 3.5	100 ± 0.5	100 ± 1.0	98.42 ± 0.5
F2C3	80.46 ± 1.5	0	65.86 ± 2.3	72.06 ± 0.7
F2C4	76.01 ± 1.8	46.47 ± 1.2	100 ± 0.5	32.04 ± 1
F2C5	52.99 ± 2.9	0	73.28 ± 3.5	82.49 ± 3.2
F2C2A*	100 ± 0.3	99.92 ± 1.3	97.97 ± 1.2	100 ± 0.8
F2C2B*	96.68 ± 1.0	99.98 ± 1.3	96.84 ± 1.0	99.18 ± 1.2

Minimum Inhibitory Concentration (MIC)

The sesquiterpene laurinterol, isolated from the active fraction F2C2A, was evaluated using the microdilution assay to establish its MIC (Table 2).

Table 2

Minimum inhibitory concentration (μg mL^{-1}) of *L. johnstonii* fractions against biofilm formation.

	B. altitudinis	*B. pumilus*	*B. subtilis*	*B. cereus*
Ethanolic extract	1.9	1.9	1.9	<0.9
F2C2A	3.9	3.9	3.9	<3.9
Laurinterol	<0.97	<0.97	<0.97	<0.97

Discussion

A biofilm is a complex, self-sustaining ecosystem where organisms exhibit cooperation and competition relationships (Nadell et al. 2016). Moreover, several studies have indicated that bacterial cells within biofilms resist various stresses that are 1,000 times greater than those observed in the planktonic form (Ashrafudoulla et al. 2019). In marine environments, biofilms can modify or mask surface topographies and properties, leading to macrofouling colonization (Qian et al. 2022). Consequently, the prevention of biofilm formation represents a safer and more optimal strategy for the inhibition of bacterial proliferation and the avoidance of all the problems associated with biofilms.

In recent decades, there has been a growing interest in marine natural products with anti-biofilm attributes. In particular, algae synthesize a range of diverse biogenic compounds, which has been identified as an effective strategy for disrupting biofilm structures and eliminating biofilms, without causing harm to other organisms within the ecosystem (Behzadnia et al. 2024). Several publications have examined the anti-biofilm activity of organic extracts. However, the majority of these studies have focused on biofilms of human pathogenic bacteria, including *Escherichia coli*, *Salmonella*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, and have not identified the active molecules (Caudal et al. 2024).

Conversely, recent research has placed a greater emphasis on the anti-fouling properties of diverse algal groups (Nag et al. 2022). Most of the antifouling metabolites isolated to date belong to terpenoids, alkaloids, and steroids (Al-Lihaibi et al. 2019). Compounds containing halogenated furanones serve as potent inhibitors of quorum sensing across a wide range of bacterial species. This inhibition prevents the formation of sessile microcolonies on both living and non-living surfaces. The red genus *Laurencia* is found in tropical zones and represents a rich source of brominated compounds with antifouling activity against mussel and barnacle larvae, such as omaezallene (Umezawa et al. 2014), aplysin-20 aldehyde, and 13-dehydroxyisoaplysin-20 (Fukada et al. 2023). From *Laurencia viridis* the compound 15,16-epoxythyrsiferol B; 15,16-epoxythyrsiferol A; 28-hydroxysaiyacenol B saiyacenol C show antibiofilm properties (Al-Lihaibi et al. 2015).

A previous evaluation of *Laurencia johnstonii* extract exhibited antimicrobial activity against strains of marine bacteria, with MIC values ranging from 0.1 to 1 μg mL^{-1} (Aguila-Ramírez et al. 2012), as well as

against pathogenic strains (García-Davis et al. 2018). In the present study, the MIC values of the ethanolic extract of *L. johnstonii* from anti-biofilm activity were found to range between 0.9 and 1.9 µg mL^{-1}. Therefore, we aimed to isolate the active compound, identified as the sesquiterpene laurinterol, with MIC values below 0.97 µg mL^{-1} against all the bacterial strains tested. The species of *L. johnstonii*, native to the Baja California Pacific coastline, was found to contain higher concentrations of laurinterol, compared to related species from the same Pacific coastal region (Arberas-Jimenéz et al. 2020). Previously, laurinterol also showed activity against some marine bacteria isolated from algal habitats (Vairappan et al. 2001). This is important because the bacterial communities within biofilm are critical to the process of larval settlement in marine environments. Therefore, inhibiting bacterial growth on surfaces is essential to preventing larval settlement of macrofouling organisms (Rajitha et al. 2020). In this context, the antifouling activity of laurinterol, along with other 13 *Laurencia*-derived compounds, was previously evaluated against larvae of the barnacle *Amphibalanus amphitrite* (EC_{50} = 0.65 µg ml^{-1}, LC_{50} = 5.8 µg ml^{-1}). Although laurinterol showed a higher EC_{50} compared to the control ($CuSO_4$), its selectivity index (LC_{50}/EC_{50}), calculated according to its ecotoxicity (EC_{50}) against the marine crustacean *Tigriopus japonicus*, was higher (Oguri et al. 2017). These studies highlight the relevance of laurinterol as a structural model for anti-biofilm studies. Moreover, it is bioavailability and a broad spectrum of bioactive properties have proven useful for structure-activity relationship analysis (Arberas-Jiménez et al. 2020). From this perspective, our study suggests that the sesquiterpene laurinterol could be a potential candidate for inhibiting marine biofilms and may provide a valuable starting point for rational design of coatings with anti-biofilm properties.

Conclusions

This study confirms the anti-biofilm activity of *Laurencia johnstonii* extract and its main compound, laurinterol, against all biofilm-forming marine bacterial strains tested. These findings not only support the potential of *L. johnstonii* as a valuable natural source of bioactive compounds with potent anti-biofilm properties but also underscore its significance in marine biotechnology and antimicrobial research. The extract and laurinterol demonstrated consistent efficacy in inhibiting biofilm formation, suggesting their potential use in preventing bacterial colonization in aquatic environments, where biofilm-related issues are common.

Furthermore, the promising results of this study highlight the importance of exploring marine resources, particularly algae, as an untapped reservoir for bioactive compounds. As biofilm formation is a major contributor to chronic infections, fouling, and other industrial challenges, identifying natural substances like laurinterol offers a sustainable and environmentally friendly alternative to traditional chemical treatments. The potential application of *L. johnstonii* extract in various industries, such as healthcare, marine engineering, and environmental conservation, warrants further investigation.

Declarations

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Competing interests

The authors declare that there is no conflict of interest.

Availability of data and materials

All relevant data are within the manuscript and supplementary material file. Any additional data is available from the corresponding author upon reasonable request.

Authors' contribution

Writing original draft preparation: MPAS. Methodology: RNAR, SGD, MMO. Data curation and formal analysis: MPAS, ALGC, SGD. Writing-review and editing: ALGC, RNAR, SGD, MMO. Project administration and supervision: RNAR, MMO.

Ethical Approval

Not applicable.

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Figures

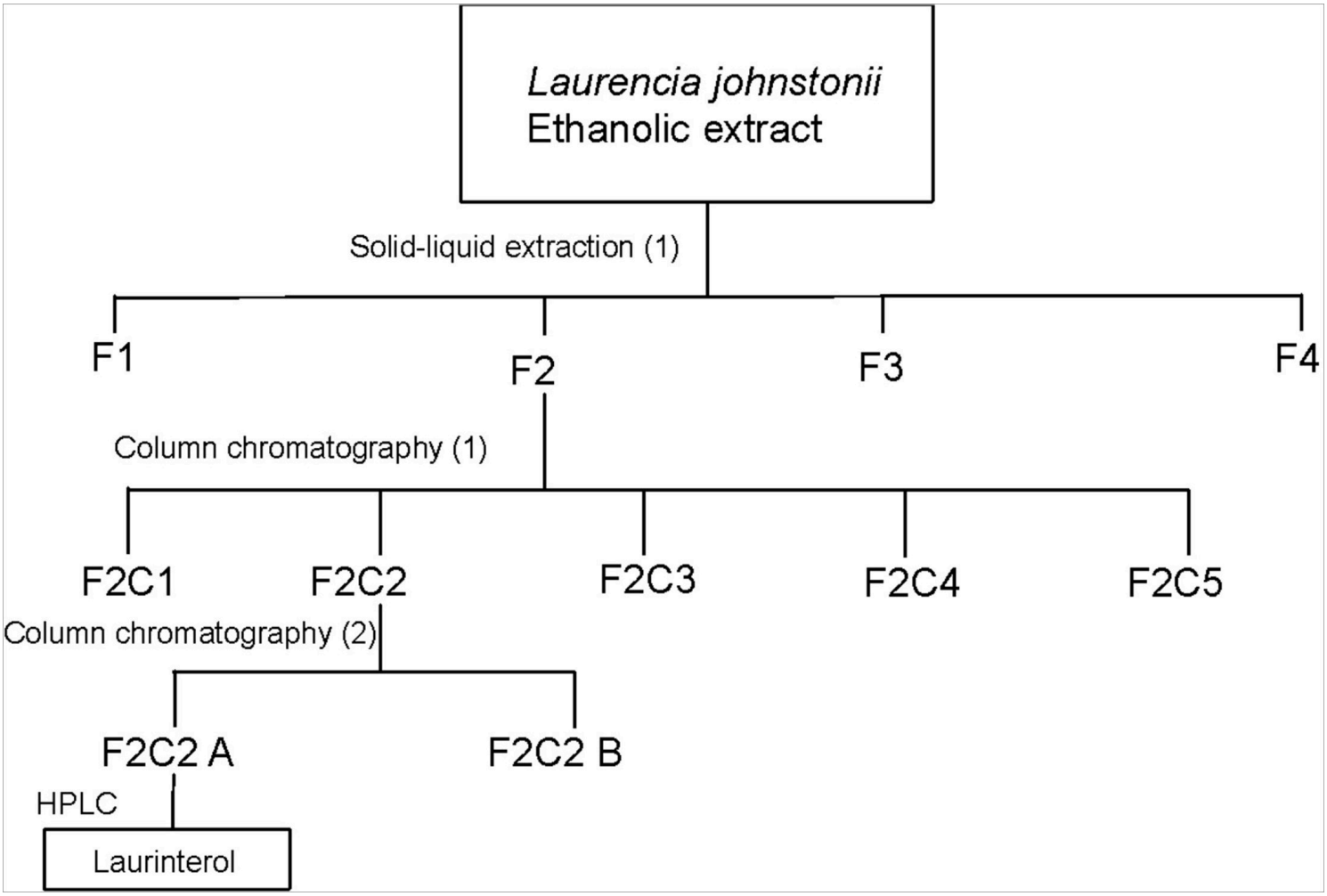

Figure 1

Bio-guided fractionation of *Laurencia johnstonii* extract to obtain laurinterol.

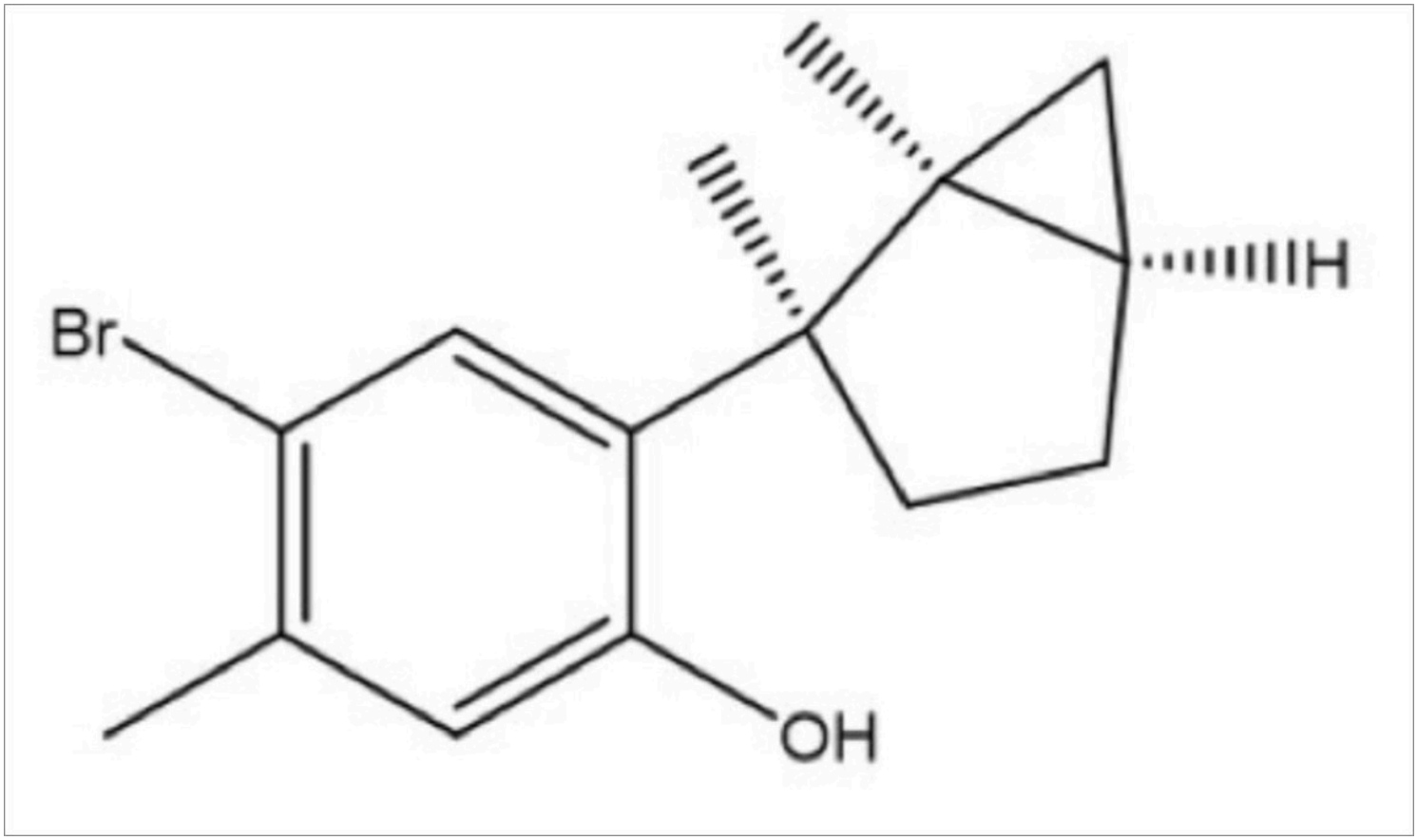

Figure 2

Laurinterol sesquiterpene isolated from *Laurencia johnstonii.*

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