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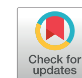
Review article

Bioprospecting marine actinomycetes for antileishmanial drugs: current perspectives and future prospects



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ABSTRACT

Revived analysis interests in natural products in the hope of discovering new and novel antileishmanial drug leads have been driven partially by the increasing incidence of drug resistance. However, the search for novel chemotherapeutics to combat drug resistance had previously concentrated on the terrestrial environment. As a result, the marine environment was often overlooked. For example, actinomycetes are an immensely important group of bacteria for antibiotic production, producing two-thirds of the known antibiotics. However, these bacteria have been isolated primarily from terrestrial sources. Consequently, there have been revived efforts to discover new compounds from uncharted or uncommon environments like the marine ecosystem. Isolation, purification and structure elucidation of target compounds from complex metabolic extract are major challenges in natural products chemistry. As a result, marine-derived natural products from actinomycetes that have antileishmanial bioactivity potentials have been understudied. This review highlights metagenomic and bioassay approaches which could help streamline the drug discovery process thereby greatly reducing time and cost of dereplication to identify suitable antileishmanial drug candidates.

1. Introduction

1.1. Leishmaniasis

Leishmaniasis, a vector-borne infection is brought about by an intracellular protozoan parasite having a place with the class Leishmania. Additionally, it is spread by the nibble of a tainted female phlebotomine sandfly (Figure 1). Three primary types of the disease have been reported in humans, which are believed to be caused by different species (among the over 20 identified/recognised leishmania species). They include cutaneous leishmaniasis (CL), which is the most prevalent and less acute type with usually self-healing ulcers and visceral leishmaniasis (VL) usually referred to as kala-azar or black fever. Later is the most severe form of the disease which manifests itself as systemic illness. It is deadly whenever left untreated in more than 95 % of the cases. The third, mucocutaneous leishmaniasis, is an uncommon kind of the illness brought about by the cutaneous type of the parasite. It is typified by the incomplete or all out obliteration of the mucous layers of the nose, mouth, and throat [1, 2].

The disease occurs frequently in tropical as well as subtropical regions of the world, particularly in Africa, Asia, The Americas, South America, and Southern Europe, where fatal epidemics have occurred. Post-kala-azar dermal leishmaniasis (PKDL), a spin-off of instinctive leishmaniasis arise essentially in East Africa and on the Indian subcontinent 5–10 % of patients with kala-azar are accounted for to come down with the condition [1]. Risk factors usually related to this disease include socio-economic conditions such as poor housing and domestic hygienic conditions, poverty, malnutrition, weak immune systems, population displacement, and environmental changes such as deforestation and urbanization. Climate change also comes into play as it affects epidemiology in several ways. The approximate number of cases range from 700, 000 to 1 million cases with an annual death rate ranging from 20 to 30 thousand deaths [1].

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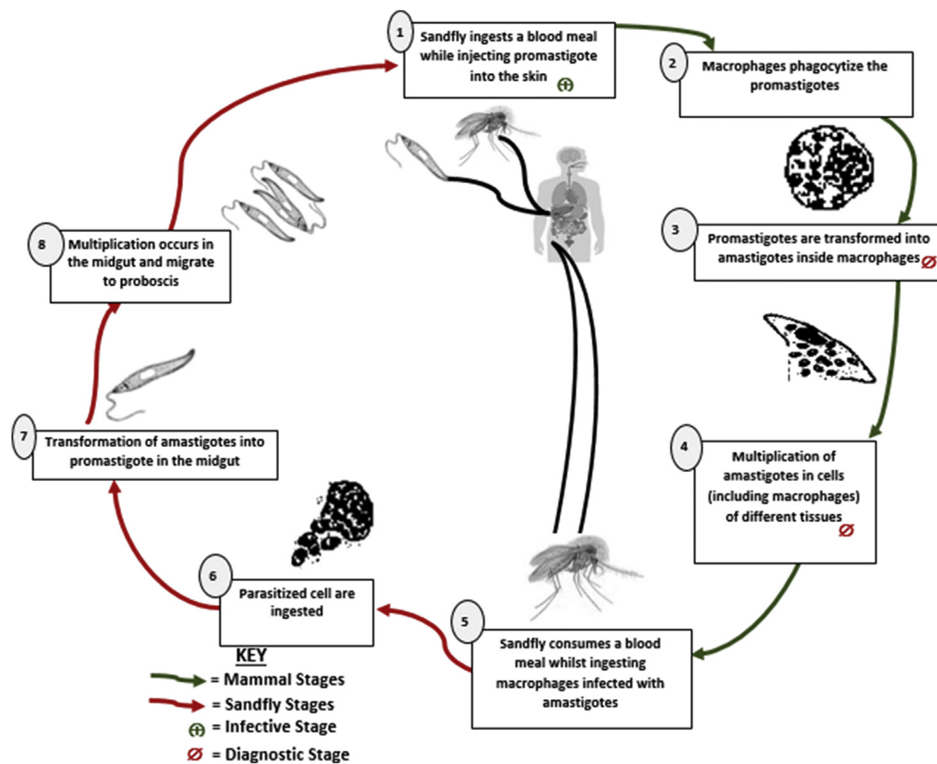

Figure 1. The life cycle of *Leishmania* sp.

1.2. Geographic distribution

Leishmaniasis is endemic in over 90 countries globally, with morbidity and mortality on a daily increase [3]. The cutaneous and visceral leishmaniasis are transmitted naturally from the endemic regions to neighboring temperate areas where vectors are present without disease prevalence [4]. Worldwide, 98 countries are reported to be endemic for kala-azar and approximately 0.2–0.4 million of new VL cases also occur annually. Over 90 % of new cases have been reported in six countries: Bangladesh, Brazil, Sudan, South Sudan, Ethiopia, and India. About 90 % of VL cases in the Indian subcontinent occur in India, particularly in the northern part of Indian state of Bihar; Bangladesh and Nepal which contribute to the rest of the disease burden in the Indian subcontinent [5]. There has however been a steady decline in the number of kala-azar cases in these countries from over 77 000 reported cases in 1992 to fewer than 7000 cases in 2016. In 2016, Nepal recorded 242 new cases with 6249 in India and 255 in Bangladesh [6]. In western Europe, France, Spain, Portugal, and Italy have been regarded as highly endemic in human and canine leishmaniasis and transmitted rapidly to northern regions [7]. Some autochthonous cases have also been observed in other European countries [8, 9]. Di Muccio et al. [10] reported a relatively low incidence of leishmaniasis importation into Italy between 1986–2012. Underreporting of imported leishmaniasis seems to be a challenge in other endemic countries of southern Europe; in Greece, only 2.8 % of CL cases from 1998 to 2011 were described with the probability of being imported [11]; also, limited number of patients were routinely recorded as a similar challenge in Spain [12]. Cutaneous South American leishmaniasis is caused by several species of leishmaniasis. Lack of appropriate treatment may lead to mucocutaneous leishmaniasis, mainly with *L. b. braziliensis* and *L. b. panamensis* [13]. In Africa, leishmaniasis is mostly endemic to countries in the North, central, east, and horn of Africa (Figures 2 and 3). In West Africa the disease is also endemic, although it appears to be understudied, less acknowledged and recorded in the region [14]. Thus, one of the reasons why it has been classified as a neglected tropical disease (NTD) [15]. In West Africa, the disease was first documented in Niger in 1911. Other cases were reported to have been discovered in Mali, Nigeria, Senegal, Cameroun, Burkina Faso, Ivory Coast, Togo, Mauritania, Gambia, Guinea, and also Ghana. In North African countries, including Tunisia, Morocco, Algeria, and Egypt, the disease is more prevalent. In East Africa, the outbreak is significantly aggravated by some factors, including poverty, conflict, and migration [16]. Data sources show that leishmaniasis burden, particularly Visceral leishmaniasis (VL) poses a serious issue to public health in the region and accounts for the second-highest VL cases next to the Indian Subcontinent [17]. Yearly, large numbers of cases and deaths are recorded, and these numbers climb higher in epidemic years which occur frequently. It is more prevalent than the other two forms: cutaneous and mucosal in East Africa and is usually caused by the parasites *L.infantum, L.donovani*, and occasionally *L.major* [17]. Areas, where the disease is endemic include Eritrea, Ethiopia, Kenya, North Sudan, Somalia, Southern Sudan, and Uganda [18, 19]. Post-kala-azar dermal leishmaniasis (PKDL), a skin complication of visceral leishmaniasis (VL) that generally affects kids aged below ten primarily associated with *L.donovani*, is most typical in Sudan where it affects 50 % of treated VL patients [20]. Cutaneous leishmaniasis is of little importance in the region as it only occurs in small endemic foci in North Sudan, Kenya, and Ethiopia [17]. In North Africa, *L.major* and *L.tropica* are the causes of zoonotic CL in many countries in the region [16]. One of the eight countries that constitute 90 % of CL in the World is Algeria [8]. *L. infantum*, the cause of visceral leishmaniasis (VL) in North Africa is transmitted by *Phlebotomus perniciosus*, and *P. longicuspis* [16]. *L.major*, which is transmitted by *P. papatasi, P. duboscqi and P. pedifer* is the leading cause of CL in the region, with *L. infantum* and *L. tropica* causing lower occurrences of the disease.

Cutaneous leishmaniasis is endemic in West Africa; in any case, the challenge of Cl is ineffectively portrayed because of dearth of information. It was referred to be endemic in country territories, yet flare-ups have happened in metropolitan zones as of late showing an adjustment of CL the study of disease transmission. *L.major* is accounted for as a reason for CL and spread by *Phlebotomus duboscqi*. *Sergentomyia darlingi* is likewise thought to assume a part in *L. major* transmission [21].

Minimal data is available about the epidemiology of visceral leishmaniasis in West Africa as it is highly understudied and underreported. The species causing VL in the region is said to be *L.infantum*. *L.donovani*, which is anthroponotic, was also found to be a cause of the human VL outbreak. *L.infantum* is found in dogs and is said to be the cause of sporadic cases in humans. The species has been implicated to most likely be involved in human infection [22]. However, the data available indicate a scarcity of the disease and most cases reported are anecdotal and sporadic. A significant cause of it, which is underreporting would point to the diagnostic challenges encountered by health centers in the region. Few parasitic diseases such as malaria and schistosomiasis, which frequently occur, can be confounded with the disease [21]. HVL has been reported in seven West African countries, including The Gambia, Niger, Nigeria, Ivory Coast, Togo, Burkina Faso, and Guinea Bissau, Gambia, Senegal, and Burkina Faso also report Canine VL. The greatest number of HVL cases have been reported in Niger, Nigeria, and Ivory Coast [22]. Mucocutaneous leishmaniasis is barely reported in West Africa [4].

People at risk include patients already who have HIV, and other chronic immunosuppressive diseases. Human Immunodeficiency Virus (HIV) and Leishmania co-infection have arisen as an extreme medical issue in Northern Africa and a reason for increased worry in West Africa. It has increased significantly as a complexity of leishmaniasis and has generated interests for recognising the disease as a predisposing factor of Acquired Immunodeficiency Syndrome (AIDS) [23]. In East Africa (Ethiopia and Sudan) approximately 70 % of grown-ups suffer from VL-HIV co-infection [24]. In Burkina Faso and Ghana, co-infection of CL-HIV has been reported, although it is generally rare in West Africa.

1.3. Treatment and control

Control and management of Leishmaniasis are centered around chemotherapy. The main drugs used for treatment are antimonials. Treatment choice is largely dependent on economic factors as most areas affected with leishmaniasis involve poor populations hence the use of cheap antimonials as medication in most areas in the absence of vaccines [25]. Over the last few decades, increased resistance to the pentavalent antimonials has been observed. This has necessitated the use of alternative treatment in the form of the orally available miltefosine (MIL), parenteral paromomycin, and amphotericin B (AmB) [26]. AmB, which is highly effective, has toxic effects which are ameliorated in its liposomal formulation. However, the potential risk of resistance has been identified in AmB, and the frequency of treatment failure in patients treated with miltefosine is of serious concern. Understanding the impacts, causes, and spread of drug resistance on treatment outcomes will enable the management of risks it imposes in the future [26]. Due to the shortage of knowledge on Leishmanial antimony susceptibility in many parts of the world, the frequency of the parasite resistance linked to treatment failure is unknown [25]. In places that rely upon antimony as the only therapy available, this data is important for addressing the danger of selecting and transmitting drug-resistant parasites. One major contributor to the emergence of drug resistance was the irregular and insufficient intake of drugs in individuals, leading to progressive tolerance. The high cost of drugs also contributed to the development of resistance as antimonials are the only affordable drugs, and poor patients who took other drugs could hardly complete dosages [27]. Coinfection of HIV/visceral leishmaniasis (VL) in patients is another potential source of the emergence of drug resistance [26]. Although newer drugs are being introduced to reduce the burden of the disease, their development is quite slow. Due to the limited pool of available treatment, drug development efforts for leishmaniasis have largely focused on drug repurposing of existing chemotherapeutics, including antimalarial (sitamaquine), anticancer (miltefosine), antifungal (amphotericin B), and antibiotic (paromomycin) drugs [28, 29, 30, 31, 32, 33, 34]. However, current treatment methods are faced with serious issues, including high cost, which limits accessibility in developing countries, safety concerns due to adverse effects and toxicity [35], poor compliance to treatment regimen due to discomfort faced during drug administration [36], treatment failure and drug resistance [26, 37, 38, 39]. In Field isolates, varied mechanisms of antileishmanial resistance have been known recently, whose elucidation can boost the look of novel chemotherapies recent medication and promote more practical molecular surveillance of resistance.

The present demand for novel antibiotics is on the increase due to the widespread antibiotic-resistant pathogens that cause life-threatening diseases such as leishmaniasis. Despite the considerable advancement in built biosynthesis of bioactive compounds, nature remains the most adaptable inventory with a vast supply of novel bioactive compounds [40, 41, 42]. The terrestrial environment have been the main sources of antibiotics, most frequently used in disease management. Many approaches related to drug discovery from terrestrial habitats in the past decades primarily brought about the isolation of previously identified bioactive natural products or close analogues. In any case, current information incredibly recommend that the marine habitat comprise an unexploited stockpile for new naturally dynamic compounds, especially antibiotics [43].

Parasites have also evolved adaptive metabolic enzymes, optimal targets of novel drug discovery and design [44, 45]. The chemotherapeutics available for leishmaniasis treatment cover the antimonials sodium stibogluconate (pentostam) and meglumine antimoniate (glucantime), but these compounds cause adverse side effects. This is a cause for concern as there is a need to explore natural sources for novel antileishmanial chemotherapeutics with much lower toxicity than available drugs. Several reports have shown antileishmanial activity of natural products majorly derived from plant sources [46, 47, 48]. Natural products, which contain an array of chemically diverse bioactive compounds, have proven to be the most important source of therapeutic agents against parasitic diseases [49]. These unique compounds have also been derived from microbial sources such as bacteria and fungi. For example, two new carbasugar-type metabolites were obtained from the filamentous fungus *Geosmithia langdonii* which was isolated from cotton textiles from Assiut, Egypt, during natural antileishmanial drug discovery efforts. The compounds showed promising antileishmanial activity against *Leishmania donovani* with IC_{50} values of 100 and 57 μM, respectively [50].

2. Marine actinomycetes as sources for antileishmanial drugs

2.1. Description of actinomycetes

Marine microbes have been known in the past decades as prolific producers of a repertoire of bioactive secondary metabolites with great potential as chemotherapeutics to fight infectious diseases. Consequently, many scientists have channelled drug discovery efforts on these natural microbial products, which possess various bioactivities such as antibacterial, antiviral, antifungal, antiparasitic, antitumor activities, among other bioactivities [51]. Furthermore, these various bioactivities exhibited by marine actinomycetes have been attributed to the production of secondary metabolites, which serve as mechanisms of survival of these strains in an unusual and extreme marine environment.

The ocean which possesses more than 70 % of the Earth's surface accommodates an excellent organic variety, representing over 95 % of the whole biosphere [52, 53]. Microbial variety makes up an inexhaustive pool of novel science, making a valuable inventory for creative biotechnology [54, 55]. Numerous valuable antimicrobials from secondary metabolites have been gotten from earthly microorganisms basically from microbial sources. In the late 1980s, there have been diminished efforts in this area attributable to the sensation that this resource has been thoroughly studied [53, 56]. Combating human pathogens challenged researchers who converted to new environments for novel pharmaceutical compounds from marine fungi as well as bacteria that appear to be the most important sources for novel antibiotic discovery because of their diversity and potential to grow very fast with high yield in bioreactors. The sea floor has been set up as an environment with extraordinary strains of microorganisms known as actinomycetes [54].

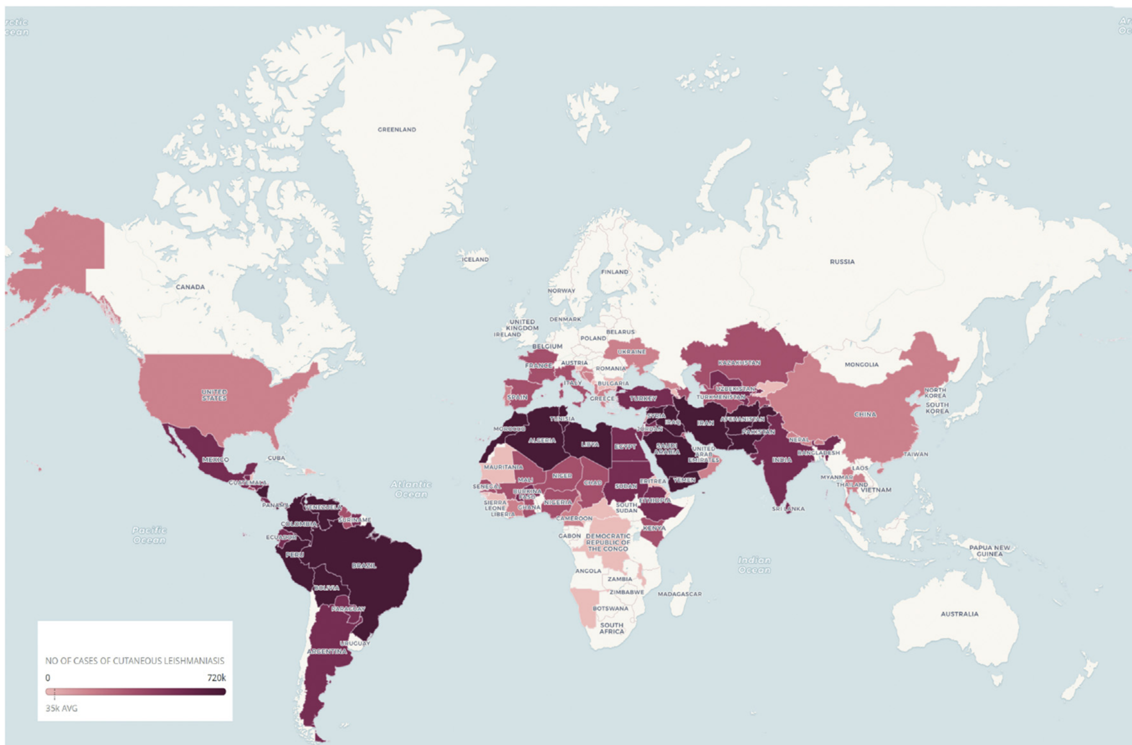

Figure 2. Map showing worldwide prevalence of cutaneous leishmaniasis. https://osu.carto.com/builder/f9bc6486-1c7d-4708-b18b-4f1c7b80c575/embed.

Actinomycetes are filamentous Gram-positive microorganisms having a place with the phylum Actinobacteria. It additionally covers one of the biggest taxonomic units among the 18 significant lineages known in Bacteria [57, 58, 59]. *Actinobacteria* populace are vast in earthbound and aquatic biological systems, explicitly in soil, where they assume an important part in reusing the tough biomaterials by separating complex combinations of polymers in dead matter such as plants, fungal constituents and animals. They likewise play out various physiological and

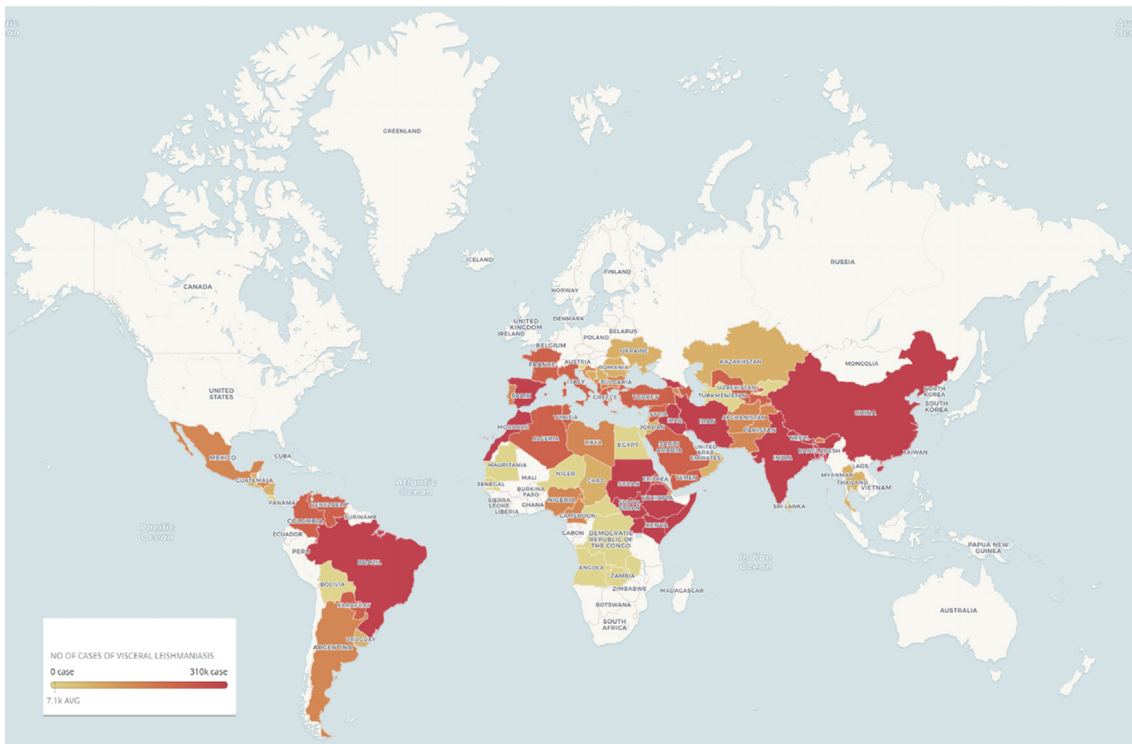

Figure 3. Map showing worldwide prevalence of visceral leishmaniasis. https://osu.carto.com/builder/0c78e7e3-b3d8-443a-b03a-e6f63854534a/embed.

metabolic properties, for example, the creation of extracellular enzymes [60, 61]. More than 23,000 bioactive secondary metabolites synthesized by actinomycetes, have been accounted for subsequently constituting 45 % of all bioactive microbial metabolites discovered among this group. Among these, *Streptomyces* provide about 7,600 compounds [55]. Several of those biosynthetic compounds are antibiotics with high bioactivities that have identified streptomycetes as the primary antibiotic-producing organism of highly valuable in the pharmaceutical industry [55]. These secondary metabolites have consequently classified into different compound groups dependent on their chemical structures, such as polyketides, polycyclic xanthones, peptides, macrolides, esters, alkaloids, trioxacarcins, lactams, terpenes and terpenoids, to mention but a few [62].

Apart from the effectiveness of actinomycete metabolites as antibacterial agents, these natural products have elicited increased interest over time as sources of highly effective antiparasitic agents. Another study reported the extraction of 400 mixtures from terrestrial organisms and subsequent to screening, uncovered ten basically assorted metabolites with high intensity with specific antitrypanosomal action [63]. A marine actinomycete *Salinispora tropica*, was reported to produce a compound Salinosporamide A also known as Marizomib, firmly restrains erythrocytic phases of human malaria parasite *Plasmodium falciparum*, most likely through interactivity with the 20S proteasome [64]. Huang *et al.* [65] also discovered four new carbolines and two new indolactam alkaloids having strong antimalarial action, o from another strain from a remote ocean South China Sea soil sediment distinguished as *Marinactinospora thermotolerans* SCSIO 00652. More recently, three antifungal macrolides were derived from *Streptomyces* sp. ISID311, a bacterial symbiont related with the fungus-growing ant known as *Cyphomyrmex* sp. These compounds showed high antiprotozoal activity against intracellular amastigotes of *Leishmania donovani* (a human parasite) recording IC_{50} values of 2.32, 0.091 and 0.073 μM, respectively, and high selectivity indexes [66].

However, bioactive compounds with antileishmanial prospects have been reported to be derived from marine actinomycetes [67]. The compound, staurosporine (Figure 4), obtained from and actinobacterial symbiont, *Streptomyces* sp. strain 11 (GU214750) which was isolated from an unidentified Mediterranean sponge was reported to possess bioactivity against *L. major* promastigotes with low EC_{50} recorded as 5.30 μM. Staurosporine, an indolocarbazole compound and known inhibitor of protein kinase C and platelet aggregation [58] has also been reported to have anticancer potentials [43]. Table 1 summarizes the antileishmanial potentials of natural products derived from marine environment.

2.2. Reports on the potentials of marine antileishmanial drugs

Several natural products with antileishmanial potentials have been derived from the marine environment. The marine organisms such as bacteria, fungi, sponges, cyanobacteria and seaweeds, synthesize various classes of compounds with unique chemical structures which have significant antileishmanial activities. Studies that reported the isolation of antileishmanial compounds from marine sources are summarized in Table 1.

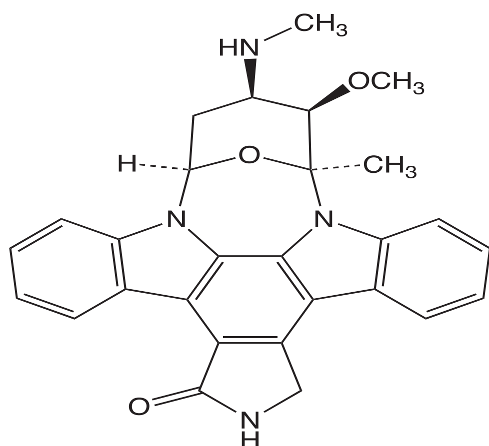

Figure 4. Staurosporine as an antiparasitic compound.

3. Strategies to assess antileishmanial activity

3.1. Culture-dependent

Members of actinomycetes from the marine habitat have been the most isolated and characterized group of microorganisms, and productive makers of bioactive secondary metabolites from Actinobacteria with interesting organic action and clinical applications [59, 98]. Therefore, more studies on isolation and the cultivation of novel and rare actinomycetes from marine and unexplored or new extreme environment will contribute to finding new bioactive compounds effective against leishmaniasis, multidrug resistant human pathogens and other diseases.

Sediments samples from deep sea or hydrothermal vent to obtain new marine actinomycetes species are obtained using specialized equipment making access to unprecedented microbial diversity a lot easier. Actinomycetes are cultivated on solid agar-based or in liquid growth medium. After introducing bacteria as starter culture, they are grown according to their unique growth and environmental conditions of where the samples were taken. Specialized growth conditions are needed to isolate and cultivate marine actinomycetes, including imitating the marine environment where the samples were gotten. The growth medium must contain sodium, which is the key medium component for the growth of novel marine actinomycetes, and it should have osmotic values similar to seawater for successful isolation [99]. The isolation media should also be supplemented with different carbon sources, combined carbon-nitrogen sources, sediment extracts, sponge extracts and natural seawater to mimic the natural environmental conditions of marine actinomycetes [100, 101, 102].

Apart from a variety of media for isolation, there are other techniques for selective isolation of rare marine actinomycetes, which is special pretreatments, such as moist and dry heat treatment, sample heating, irradiation, phenol treatment, microwave, calcium carbonate treatment and bacteriophage in order to eliminate unwanted microorganism [103]. Overall, the isolation medium must be a low nutrient medium for effective isolation of marine actinomycetes [104]. Several selective isolation procedures should use distinct isolation media with varied elements, and also the media should limit the cultivation of unsought microbes [103]. In addition, growth inhibitors, antibiotics or other supplements for optimal growth ought to be added to the culture media to forestall the development of Gram-negative bacteria and fungi which serve as contaminants [105]. In 2013 and 2019, various isolation media and diversity of different marine actinomycetes for bioactive compounds were described from 2007 to 2017 and reviewed [98, 106]. However, isolation and cultivation of bacteria with high bioactivity potentials are hindered by drawbacks including de-replication. Eliminating the possibility of re-isolation of known bacteria and their metabolic compounds and focusing only on those that are potentially novel, is very important for novel antileishmanial drug bioprospecting.

However, the isolation and cultivation of some marine actinomycetes that are viable but not culturable are strenuous and challenging, because they have a complex nutritional/environmental requirement. Consequently, novel bacterial cultivation platforms have been developed (iChip and hollow-fiber membrane chamber - HFMC) for in situ cultivation. This has broadened the scope of natural product discovery where bacteria which were thought to be previously uncultivable were successfully isolated, and their bioactive molecule characterized utilizing these techniques [107, 108, 109, 110].

The HFMC, a cultivation device for environmental microorganisms, consists of injectors connecting items of permeable hollow-fiber membrane (48–96 pieces). A chamber unit is made of porous hollow fiber

Table 1. Antileishmanial drugs derived from the marine environment since 2010.

Source	Class	Bioactive compound	IC_{50}	References
Marine Sponges				
Pandoras acanthifolium	Steroid	Pandaroside	0.051 μM	[69]
Agelas mauritiana	Alkaloid	Ageloxime D Ageloxime B	29.28 μg mL^{-1} 28.55 μg mL^{-1}	[70]
Agelas conifera	Alkaloid	Hymenidin	29.87 μg mL^{-1}	[71, 72]
Axinella verrucosa	Alkaloid	Bromoaldisin	>90 μg mL^{-1}	[71, 72]
Ircinia spiculosa	Alkaloid	Tryptophol	9.6 μg mL^{-1}	[73]
Plakortis simplex	Polyketide	Simplexolide B	13.82 μg mL^{-1}	[74]
Spongia sp. and *Ircinia* sp.	Terpene	Furospinulosin-1	14.2 μg mL^{-1}	[73]
		Furospongin-1	4.8 μg mL^{-1}	
		Heptaprenyl-*p*-quinol	18.9 μg mL^{-1}	
Tedania braziliensis	Alkaloid	Pseudoceratidine	NA	[75]
Dysidea avara	Quinone	Sesquiterpene avarone and avarol	*L. infantum* amastigote (7.64 and 3.19 μmol/L) and promastigotes (28.1 and 7.42 μmol/L), and *L. tropica* promastigotes (20.28 and 7.08 μmol/L)	[76, 77]
Marine Bacteria				
Paenibacillus polymyxa	Alkaloid	Paenidigyamycin A	*L. major* (0.75 μmol/L) and *L. donovani* (7.02 μmol/L)	[78]
Streptomyces sanyensis	Alkaloid	*Indolocarbazole staurosporine*	*L. amazonensis* promastigotes and amastigote (0.06–10.65 μmol/L), *L. donovani* promastigotes (0.50 – > 40 μmol/L)	[79]
Streptomyces sp. strain 11 (GU214750)	Alkaloid	Indolocarbazole staurosporin	*L. major* promastigotes with EC50 5.30 μM	[67]
Streptomyces sp. VITBVK2	unknown	Crude extract	100 μg/ml against amastigotes in infected J774A.1 macrophages	[80]
Streptomyces sp. EIIB strain	coumarins	Crude extract	15 000 μg/mL for Leishmania (Viannia) peruviana and Leishmania (V.) braziliensis.	[81]
Marine Cyanobacterial				
Lyngbya majuscula	Peptide	Dragonamide E	5.1 μM	[82, 83]
Lyngbya majuscule	Peptide	Dragonamide A	4.25 μg Ml^{-1}	[84]
Lyngbya majuscula	Peptide	Almiramides A–C	2.4 μM	[84]
Schizothrix	Peptide	Gallinamide A	9.3 μM	[85]
Oscillatoria nigro-viridis	Peptide	Viridamides A, B	1.5 μM	[83, 86]
Oscillatoria sp.	Polyketide	Coibacin A –D	0.68–4.99 μg mL^{-1}	[87]
Marine Algae				
Bifurcaria bifurcate (Brown alga)	Terpene	Bifurcatriol	*L. donovani* amastigotes (18.8 μg/ml)	[88]
Dictyota spirali	Terpene	Spiralyde A	*L. amazonensis* promastigotes (15.47 ± 0.26 and 36.81 ± 5.20 μmol/L)	[89]
Cystoseira baccata	Terpene	tetraprenyltoluquinol and tetraprenyl	*L. infantum* promastigotes and amastigotes (44.9 ± 4.3 and 94.4 ± 10.1 μmol/L)	[90]
Stypopodium zonale	Terpene	Atomaric acid and its methyl ester derivative	*L. amazonensis* intracellular amastigotes (20.2 and 22.9 μmol/L)	[91]
Laurencia viridis (red algae)	Terpene	Oxasqualenoid metabolites	Promastigotes of *L. Amazonensis and L. donovani* (5.40–46.45 μmol/L)	[92]
Marine Fungi				
Eurotium repens	Polyketide	Tetrahydroauroglaucin	22 μg mL^{-1}	[93]
		Flavoglaucin	23 μg mL^{-1}	
		Auroglaucin	7.5 μg mL^{-1}	
Paecilomyces sp. 7A22	Lactone	Harzialactone A	*L. amazonensis* promastigotes forms (5.25 μg/ml) and intracellular amastigotes (18.18 μg/ml)	[77, 94]
Marine seaweed				
Laurencia dendroidea (Red seaweed)	Terpene	Obtusol	6.20 μg mL^{-1}	[95]
Canistrocarpus cervicornis (Brown seaweed)	Terpene	(4R,9S,14S)-4α-Acetoxy-9β,14α-dihydroxydolast-1 (15),7-diene	2 μg mL^{-1}	[96]
Dictyota pfaffii (seaweed)	Terpene	Dolabelladienetriol	14.16 μg mL^{-1}	[97]

polyvinylidene fluoride (PVDF). The injection and sampling are kept sterile (capping with a cover) during cultivation. Microbial samples are serially diluted and then injected into the chamber. Thus, the chamber can be kept in an engineered environment for the specified incubation time. In contrast, the membrane area of HFMC remains soaked in the liquid section throughout the growth period. This allows an exchange of chemical compounds (metabolites and nutrients) yet, prevents the escape of microbial cells. Therefore, various types of pure cultured cells is cultivated in each chamber and maintained under appropriate conditions which simulate the environment of initial microbial isolation [107].

The iChip method utilizes an arrangement of numerous little circulating chambers, each loaded with one cell. The iChips comprises of a focal hydrophobic plastic polyoxymethylene (POM) plate with a variety of openings (1mm in distance across). The dispersion framework allows the cells on the iChip to move with accessible supplements and conditions in the natural environment [111]. In order to isolate microorganisms using this technique, soil or sediment samples are suspended in dilute molten growth medium to ensure that one cell is set for every through-hole. The molten medium solidifies and inhibits the movement of the cells in the iChip. Thus, to avert cell migration, polycarbonate membranes are applied to both sides of the focal plate thereafter. Cover plates with coordinating through-openings are then screwed to seal the framework and hold the membrane set up. This is done as an impediment to the cell. This strategy has appeared to create an increased number of novel microorganisms than the conventional technique which requires the use of petri-dish [108].

Different in vitro methods for assessing the antileishmanial activity of bioactive secondary metabolic extracts/compounds include classical methods *in vitro* including direct counting and MTT assays. These methods use axenic promastigotes or amastigotes as an affordable option for evaluating huge secondary metabolite libraries [112]. The extracellular variety of parasites known as promastigotes are resident in the gut of the sand fly insect while amastigotes are the parasite forms that stay for long periods in the infected host. They infect cells recruited to lesions and spread the infection to some other secondary regions in the host [113]. For other *in vitro* assays such as the use of macrophages, amastigotes are the appropriate form of the parasite to use. The convenience of parasite maintenance in culture and assay performance permits the evaluation biological activities of numerous mixtures utilizing unsophisticated hardware [68]. Other screening techniques applied to antileishmanial drug discovery process include reporter gene technology, acid phosphatase activity, radioactive nucleotides and *ex vivo* techniques such as high content assays and classical *in vivo* analysis (Table 2).

3.2. Culture-independent

The number of microorganisms that have been isolated using traditional culturing techniques is less than 1 % of microorganisms, leaving the substantial microorganisms and their secondary metabolic pathways unavailable [137, 138, 139]. The development of metagenomic and culture-free strategies have provided different instruments to decide the level of the yet-to-be-cultured microbial variety in full and enabled admittance to the secondary metabolic pathways in these uncultured microorganisms [140, 141]. Metaproteomic and metagenomic technologies enable newer approaches to gene, genome, protein and metabolic pathway discovery [138]. However, the number of yet-to-be-cultured marine microbiota synthesizing secondary metabolites has greatly reduced accessibility to an enormous genetic diversity concerning unexplored chemical resources required in therapeutic and pharmaceutical applications [54, 142]. This contains complex sponge, tunicates, microbes from marine and terrestrial consortia in which case the presence of huge populations of diverse microbiota as well as their related genomes which bear natural product gene clusters remain undiscovered [138, 139]. This novel supply of chemical and metabolic variety will help increment fundamental data and add to momentum drug discovery endeavors against a few illnesses [138].

With the emergence of infectious diseases and the increase in drug-resistant pathogens, continuous search for new technologies must hasten new drug discovery processes for different diseases. Culture dependent techniques have been used for several years to select microbial strains for antibiotics production. Various culture media for cultivating microbes from marine environment are obtainable, but a huge section of the cluster remains underexplored resulting from impediments associated with in-vitro culturing, inadequate in-depth taxonomy, and physiological characterizations [143]. Actinomycetes have been isolated from environmental sources for several years. However, with the recent establishment of metagenomics, a culture independent technique which explores the genomic sequences for biosynthesis potential of drugs as well as its gene expression from several unculturable microbes and marine actinomycetes have been boosted remarkably [144, 145].

Metagenomics tool gives a deep view knowledge of the complete genome obtained from a mixed population of microorganisms and provides immediate access to the bioactive prospects of the microbial consortia without getting a pure culture. Metagenomic techniques involve robust data analysis while searching for novel bioactive molecules, and other molecules with potential importance in the pharmaceutical industry. Consequently, this helps to fast track the deduction of the cellular and metabolic pathway of the strains involved in the biosynthesis of unique bioactive secondary metabolites [146]. Furthermore, in metagenomics, secondary metabolite biosynthesis genes may be amplified or synthesized and heterologously expressed in an applicable well-characterized bacterial host. Therefore, use of metagenomic tools can transform the process of more drug discovery from the marine environment to treat leishmaniasis.

3.2.1. Community fingerprinting

However, the culture-independent molecular approach has however confirmed the presence of indigenous actinobacteria through different studies [147, 148, 149]. These culture-independent strategies focus on nucleic acids directly extracted from the samples [150]. They typically involve the amplification of DNA derived from environmental samples with PCR the future assessment of the actinobacterial diversity based on the amplified DNA (community fingerprinting) [142]. The amplified genes could otherwise be cloned, sequenced and the novel actinobacteria available in the sample identified and enumerated [151, 152]. Monciardini *et al.* [142] developed highly selective primer sets for PCR amplification of the16S rDNA from the Actinomycetales families *Thermomonosporaceae, Streptosporangiaceae, Streptomycetaceae,* and *Micromonosporaceae* as well as from the genus *Dactylosporangium*. All primer sets, evaluated based on genomic DNA from reference organisms, indicated elevated specificity and sensitivity. However, the use of the primers for environmental samples indicated the presence of these groups of actinobacteria in high frequency and showed sequences that could be related to novel actinobacterial [147]. Other molecular techniques for example, terminal restriction fragment length polymorphism (T-RFLP) analysis, evaluates the size polymorphism of terminal restriction fragments from a PCR-amplified marker [153] and the method combines at the minimum, three technologies such as PCR, electrophoresis and comparative genomics/RFLP. Denaturing gradient gel electrophoresis (DGGE) as well as TGGE (thermal-GGE) are also molecular methods such that fragments of DNA of different sequences but equal length may be resolved electrophoretically [153]. In this case, separation of DNA fragments is based on decrease in electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels which contains a linear gradient of a denaturing reagent or a linear temperature gradient. PCR-DGGE strategy was broadly used to affirm the variety of actinobacteria present in the environmental ecosystem [139, 148, 153, 154].

Table 2. Bioassays for evaluating natural products/compounds against *Leishmania* sp.

Assay type	Principle	Advantages/disadvantages	References
Direct counting method	This assay uses haemocytometer and microscope. Sample is incubated with test compound in a 96-well microplate for 24–96h and viewed with microscope	The technique allows for screening of large amounts of compounds using inexpensive equipment. A major disadvatage of the assay is that it is time-consuming and inaccuracy in EC_{50} values	[68, 112]
MTT colourimetric assay	Based on oxidative activity of mitochondria and used for HTS screening	High reproducibility and accuracy of results, however oxidation of the MTT substrate with test compounds results in false-negatives.	[114, 115]
Alamar blue assay	It is used as an alternative to MTT assay substituted with alamar blue indicator. Living cells are metabolically active and reduced	This assay is simple, reproducible and reliable	[116, 117]
Intracellular assay	a) It involves the use of mammalian cells e.g. macrophages infected with *Leishmania* promastigotes	For most Leishmania sp., it is difficult to maintain them for an adequate period of 96–120 h because of oxidative mechanisms which lead to elimination of effective macrophages	[118]
	b) It involves the use of macrophages infected with *Leishmania* amastigotes	Results are accurate and highly reproducible after 96–120 h. However, it is time consuming since amastigotes are first isolated from infected mammals	[68, 119]
Reporter gene technology	This involves the use of reporter gene including firefly luciferase gene to create modified parasites with an easily quantifiable phenotype that is easily distinguished	It is high through put, generates objective quantitative data and requires minimal manual labour.	[68, 120]
Radioactive nucleotides	These methods involve the use of ^{3}H thymidine in the assessment of *Leishmania* promastigote viability	The test is highly sensitive but generates radioactive waste and time consuming	[121, 122]
Flow cytometry	This method requires the evaluation of Leishmania promastigotes viability with stains including SYBR-14, 5,6-carboxy-fluorescein diacetate succinimidyl ester (CFSE)	This method requires drug incubation period of not more than 24 h	[123, 124, 125]
Acid phosphatase activity	This method is based on an enzymatic hydrolysis of p-nitrophenyl-phosphate	The assay is very simple highly reproducible and cost effective although its use based on literature report is limited	[126]
HTS	This process is an automated process used to screen large compound libraries against biological targets for large scale data analysis	It enables fast screening of small molecules based on genomics and combinational chemistry. However, difficulty of obtaining compound libraries and inadequate genetic or chemical limits improvement in this method	[68, 127]
Selectivity Index (SI)	This involve the correlation between antiparasitic activity and invitro cytotoxicity (EC_{50} against mammalian cells/EC_{50} against leishmania)	A large panel of cells with candidate compound are screened to evaluate their invitro efficacy	[68]
Multi-species phenotypic High Content Screening (HCS)	This method is based on the infection of phorbol 12-myristate 13-acetate (PMA) differentiated THP-1 macrophages with the stationary-phase promastigotes	The assay is robust and quite applicable to drug discovery efforts for leishmanisis associated with different species. However, species of choice should be priotized and introduced early for easy selection of broad-spectrum compounds	[128]
Ex-vivo	This assay combines biologically important phenotypic High Content Assay (HCA). It is based on primary macrophages infected with virulent, animal-derived amastigotes and inviability assays on promastigotes obtained from dermatrophic and viscerotrophic *Leishmania* species	The assay easily evaluates important stage and species-specific differences in compound efficacy	[38]
Classical *in-vivo*	This assay uses parasite burden detection technique through animal euthanasia at various points after infection	It is time-consuming, laborious and inapplicable for automation	[129]
Real-time *in vivo*	The method involves infection of animals with bioluminescent transgenic *Leishmania* for parasite burden quantification using fluorescence intensity	It is faster, more accurate and sensitive image-based technology	[130, 131, 132, 133, 134, 135, 136]

3.2.2. Next-Generation Sequencing

Next-Generation Sequencing (NGS) is more desired for drug discovery because they ease the search clusters of the genes of interest identified as essential genes for synthesis of numerous bioactive compounds in marine actinobacteria e. g. type I, II, and III PKSs (Polyketide synthases), Massively Parallel Signature Sequencing (MPSS) and NRPS (Non-Ribosomal Peptide Synthetase) [155]. Genome sequencing and analyses reveal several gene clusters for the biosynthesis of secondary metabolites (BGCs) distinctive for isolates. Random sequencing (Shotgun sequencing) and target sequencing techniques are the two main approaches used to explore genes or gene clusters responsible for producing novel bioactive metabolites. Shotgun sequencing successfully recovers total genomic DNA from marine environments without culturing. The technique requires the isolation of high-quality genomic DNA followed by fragmentation of genomic DNA into smaller pieces randomly followed by sequencing for several rounds. The last step is re-assembling by multiple overlapping of sequenced segments for the whole sequence with bioinformatic tools. This technique has been used to characterise the genome of microorganisms including their metabolic cycles, genetic content, and secondary metabolites [156]. However, selective identification of the active groups responsible for novel bioactive metabolites is lacking in this technique.

Targeted metagenomics technique aims to sequence environmental DNA pools through adoption of high-throughput sequencing approach to limit genetic complication. Data collection on the genetic structure and constituents, required evaluating the mode for metabolic adaptive features for any specific gene or gene cluster associated with bioactive prospects [157]. In addition, metagenomic techniques for drug discovery have been broadly classified as function- or active-based screening and sequence or homology-based screening [158].

Sequencing with the homology-based screening approach is largely determined by accurate amplification of comparatively huge DNA fragments, use of acceptable DNA assembly techniques, and host selection.

3.2.3. Function or activity-based screening

This method is used to detect bioactive metabolites with the help of generating genomic libraries derived from environmental samples and subsequent screening for the direct evaluation of the specified bioactive compound. This will allow identifying and discovering of new classes of bioactive compounds with antileishmanial and other antiplasmodial, antibacterial, antifungal, antiviral and antitumor activity, which were not detected initially [159]. This screening can be achieved through direct detection of appropriate gene phenotypes, heterologous relatedness and substrate-induced gene expression. In addition, recent advancements in the fields of metagenomics, novel methods of isolation of marine actinomycetes and recent techniques of cultivating previously uncultivated actinomycetes, will pave way for new possibilities on leishmaniasis drug discovery.

3.2.4. Strain prioritization

Other metagenomic approaches to novel drug discovery from natural products include strain prioritization and genome mining of natural products of a specific group of compounds. For example, Ben Shen's group, from the Scripps Research Institute, Florida, U.S.A., fostered a high-throughput ongoing PCR strategy for strain prioritization to identify the most encouraging strains from a microbial strain assortment for natural product disclosure [160]. The same research group in 2016 also surveyed the TSRI actinomycetes collection for their enediyne PKS gene cassettes to identify new enediyne-producers from actinomycetes. From 3,400 representative strains obtained in their study, 81 unique enediyne-producers were detected based on the identity of the enediyne PKS gene cassettes taxonomy including geographic locations of the isolated actinobacteria [160]. This real-time PCR method could be adopted to rapidly screen and identify antileishmanial metabolite producers based on specific PCR products, in an exceedingly high-throughput manner through melting curve analysis and confirmation by gel electrophoresis and DNA sequencing.

4. Conclusion

Natural products and their relatives continue to be approved as new medications. With over 150,000 known small molecules derived from natural sources, it is apparent that earlier natural products could often be re-isolated in the course of bioassay-guided fractionation. The marine environment has proven to be a prolific resource where antileishmanial compounds could be derived from invertebrates such as sponges, algae and microbes including actinobacterial symbionts associated with marine plants and animals. Actinobacteria possess a unique biosynthetic mechanism which have produced many unique bioactive compounds from which chemotherapeutic agents are derived and currently used in the treatment of many diseases. However, information about actinobacterial natural products with antileishmanial bioactivity is limited. Further studies are therefore necessary in the area of drug discovery of diverse strains of marine actinomycetes from underexplored environments which tend to contain several actinobacterial strains in many largely untapped environments which remain yet to be exploited.

Information obtained from studies related to culture-independent procedures to assess the presence and distribution of actinomycetes in the marine environment could also be used to design selective isolation techniques enabling efficient isolation of a diverse novel taxa of marine actinomycetes. In addition to this, the increased studies are encouraged to improve more rapid and low-cost bioassay approaches for HTS of antileishmanial drug leads of natural microbial products therefore leading to discovery of novel compounds that would in the long run contribute immensely to antileishmanial drug discovery.

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