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BIOACTIVITY-GUIDED ISOLATION OF METHYL GALLATE AS AN ACTIVE ANTILEISHMANIAL AGENT FROM *ACCACIA NILOTICA*

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Abstract

The lack of ideal treatment of the fatal disease Leishmaniasis necessitates discovery of new drugs. Medicinal plants have been an efficient source for parasitic drug discovery. Bioactivity guided fractionation of methanolic extract of the pods of *Accacia nilotica* resulted in isolation of a white solid compound. The *invitro* antileishmanial activity of the isolate was assessed against *Leishmania donovani* promastigoates using AmBisome as a standard drug and the resultant IC50 and IC90 values were 4.8 and 8 µg/ml, respectively. Phytochemical study of the isolate indicated the presence of poly hydroxyl functionalities. Spectral data (IR, NMR and Mass) proposed that the isolated compound was methyl gallate.

Keywords: *Leishmania, Acacia nilotica, Bioactivity guided fractionation and Methyl gallate*

Introduction

Leishmaniases are group of neglected tropical diseases. They are vector-borne infection caused by protozoan parasites of the genus Leishmania (1). Three clinical forms of Leishmania infection, namely, visceral, cutaneous and mucocutaneous, are recognized in humans and they are caused by different Leishmania species (2). The most life-threatening clinical form is visceral leishmaniasis (VL), which is fatal if untreated, and is mainly caused by *Leishmania donovani* (3). VL affects 200 to 400 thousand people worldwide annually. Ninety percent of the VL occurs in Sudan, Ethiopia, Bangladesh, India, Nepal, and Brazil (4,5). Treatment of Leishmaniasis has always been problematic; it is based on pentavalent antimonial drugs, pentamidine, paromomycin and amphotericin B (Figure 1). These drugs are toxic and prone to drug resistance. In addition, the lack of efficacy, high cost, parenteral route of administration affecting compliance and lack of access in regional areas have contributed to treatment failure (6,7). This has mandated discovery and development of new drugs that would possibly be a key for safe, effective and affordable antileishmanial treatment (8). As a potential source for drug discovery, medicinal plants have always played a key role in curing diseases (9). *Accacia nilotica* (L.) is an important medicinal plant of tropical and sub-tropical regions. The plant belongs to family *Fabaceae* of genus *Acacia* and it is a source of many active secondary metabolites which may serve as potential candidates for drug development with greatest possibility of success in near future (10). In this context, the antileishmanial activity of three organic solvent extracts and water residue of the plant were tested *invitro* against *Leishmania donovani* promastigotes (11). The study revealed that the ethyl acetate (EtOAc) extract showed promising antileishmanial activity. Nevertheless, no attempts were made to isolate and characterize the active compounds. The current study aimed to isolate and characterize the active compounds of the EtOAc extract following a bioactivity guided fractionation approach.

Methods

General

Column chromatography was carried using a column (ultra pack glass (26X300mm), packed with SI40B silica from Yamazen corporation). The fractions were detected using UV-D2 UV monitor and Refractive index which was recorded using Shimadzu RID-10A refractive index detector. TLC detection was carried using pre-coated TLC plates (silica gel 60F254 aluminum sheets Merck-Germany). TLC plates were detected in a UV detector with UV absorption around 254nm. IR spectrum was recorded on a Nexus670NT FT-IR and is reported in frequency of absorption (cm^{-1}). NMR spectra were recorded on a Varian Gemini 300, 500MHz and JEOL JNM-AL400 MHz for ^{1}H and ^{13}C, respectively. High resolution mass spectra were obtained on JEOL JMS-700N for electron impact ionization or on JEOL JMS-T100TD for electrospray ionization fast atom bombardment Mass spectroscopy using Xenon as bombarding gas.

Plant material

The pods of *Accacia nilotica* were collected from Sinar State, Sudan and taxonomically identified in comparison with authenticated samples at the Department of Pharmacognosy Faculty of Pharmacy University of Khartoum, Sudan. The seeds were removed and the airdried husk was crushed and powdered.

Extraction

The prepared plant powder was extracted overnight using 80% aqueous methanol (MeOH). The methanolic extract was concentrated, diluted with water and fractionated in a separatory funnel using hexane, dichloromethane (DCM) and EtOAc in the named order. The EtOAc crude extract was eluted through a column (ultra pack glass (26X300mm), packed with SI40B silica from Yamazen corporation) using solvent system of hexane and EtOAc in increasing polarity manner. Collected fractions were subjected to TLC and the major component was isolated and structurally elucidated.

Invitro antileishmanial activity

The cytotoxicity and inhibitory effect of *Acacia nilotica* crude extract on the growth of clinically isolated *L. donovani* promastigotes were carried out as described previously (11). Briefly, promastigotes culture was harvested at 1200rpm for 10 min, the precipitated promastigotes were washed with normal saline and resuspended in RPMI1640 media. The promastigotes concentration was adjusted to 3x106/ml. Then, 50µl were added to a row of cells of the 96 well (Nunc) microtiter plate containing 200µl

of each test extract concentration, and a control rows of cells containing either 200µl of plane RPMI1640 media only (negative) or liposomal amphotericin B (positive). The parasites were left to multiply at 24±2˚C for 24 h, then harvested and counted in a haemocytometer chamber.

Results and discussion

Being the most active (11), the EtOAc extract was purified by column chromatography and subjected to TLC separation to reveal a single spot. The appearance of a single TLC spot, despite the varying solvent system used, was taken as an evidence for the purity of the isolate. This was further confirmed by HPLC analysis where the isolate's chromatogram showed a single principal peak at Rt value of 3.28 minutes. Structural elucidation using UV, IR and NMR spectroscopies proposed methyl gallate (Figure 2) as the possible isolate where obtained spectroscopic data was in agreement with that revealed in the literature.

The UV spectrum of the isolate showed strong absorption band at 275nm suggesting the presence of aromatic moiety having auxochromes. This finding agrees with the UV spectrum of a galloyl ester as reported by Abou-Zaid and Nozzoliollo (12).

The IR spectrum obtained in a KBr discs (Figure 3) showed broad absorption band at 3500-3200 cm^{-1} characteristic to hydroxyl functionalities with intra hydrogen bonding. A (-C-H) absorption band at 2950cm^{-1} was observed. A band at 1750-1650cm^{-1} indicated the presence of –C=O group. The band at 1650-1600cm^{-1} proposed the presence of aromatic C=C. The structure of the isolate was proposed to contain a methyl (-CH_3) as a characteristic band at 1450-1400cm^{-1} was recognized. Multiple absorption frequency ranging from 1300-1000cm^{-1} pointed to the presence of –C-O bonds of varied chemical environment. Sharp peak at 770cm^{-1} indicated the presence of a methine moiety (C-H).

^{1}H NMR (MeOD) spectrum (Figure 4) showed two strong singlets at 7.1 and 3.8 in the ratio of 2:3 indicating the presence of two aromatic protons and methoxy protons, respectively. The integration of the singlet peak at the aromatic region (7.2) for two protons pointed to the presence of two identical aromatic protons at C2 and C5 and were assigned to as the two galloyl protons. While the long singlet up filed at the aliphatic part of the spectrum 3.8 was believed to be due to the three methoxy protons, which were slightly shielded due to the adjacent ester carbonyl group.

^{13}C NMR spectrum (Figure 5) exhibited 8 peaks with different heights which were taken as an evidence for the presence of 8 carbons in a diverse chemical environment. The singlet signal at 169 was attributed to the deshielded downfield C=O carbon. The signal at 140 was assigned to the oxygenated para-aromatic carbon. The height of the peak at 146 was an indication of two symmetrical oxygenated aromatic carbons located at meta positions. The aromatic carbon next to carbonyl group resonated at 121. The two symmetrical nonsubstituted aromatic carbons at ortho positions showed a peak at 110. The most up field signal at 52 was due to the aliphatic methyl group carbon. Tatsuya et al. reported characteristic HMBC correlations for gallic acid (13).

The interpretation of the HMBC spectrum of the isolate (Figure 6) demonstrated similar correlations with those observed for gallic acid. To this end, the spectrum showed correlation between the aromatic protons' signals and the carbon of ester carbonyl moiety. A ^{2}J correlation was observed for the methine aromatic protons resonating at 7.1 with the two symmetrical oxygenated aromatic carbons resonating at 146 on the one hand and with the quaternary aromatic carbon signal at 121 on the other hand. In addition, both the carbon of the ester carbonyl (169) and the oxygenated para-carbon (140) appeared to have ^{3}J correlations with these methine aromatic protons. The HMBC spectrum also demonstrated ^{3}J correlation between the aromatic methine carbon (110) and the aromatic proton on the corresponding side of the ring. The presence of a methyl ester was clearly indicated by the ^{3}J correlation between the methoxy protons signal at 3.8 the carbon of the ester carbonyl carbon signal at 169.

Both ESI (Figure 7) and TOF Mass-spectroscopy mass spectrum showed a molecular ion peak at 185 m/z+1 (M^{+}) which agreed with the protonated methyl gallate molecule. Having not carried out elemental analysis for the isolate, it was decided to do FAB-MS instead where (M+23), corresponding to the sum weight of isolate molecular ion and sodium ion, could be detected. To this end, FAB-MS spectrum showed an abundant peak at 207

indicating that the molecular weight of the isolate was 184.

Based on the aforementioned spectroscopic interpretations, the antileishmanial isolate was identified as methyl gallate. This was further confirmed by comparison of its spectroscopic data with those reported for methyl gallate isolated form different plants (14,15).

With the isolated methyl gallate in hand, its antileishmanial activity was now investigated and it showed promising activity with Ic50 and Ic90 values of 4.8 and 8µg/ml, respectively. Both the promastigote and the amastigote forms of *Leishmania* can initiate infections. Nonetheless, when infections are initiated with promastigotes, these parasites transform into amastigotes over a period of 24 to 72 h (16). Therefore, the antileishmanial activity of tested compounds should be evaluated against the intracellular amastigotes as well (17,18). Interestingly, literature revealed that methyl gallate has a promising activity against *Leishmania donovani* amastigoates (15). Taken together with the findings, one can attribute the efficacy of the medicinal plant *Acacia nilotica* against leishmaniasis to, at least, the presence of methyl gallate.

In conclusion, bioactivity guided fractionation of the crude EtOAc extract of Acacia nilotica led to isolation of an antileishmanial compound. Spectroscopic data of the isolate was in agreement with that reported for methyl gallate. Thus, the traditional use of Acacia nilotica for leishmaniasis could be attributed, at least to, the presence of methyl gallate. The obtained findings support further the reported antileishmanial activity of methyl gallate as a promising candidate for developing new leishmanicidal agents.

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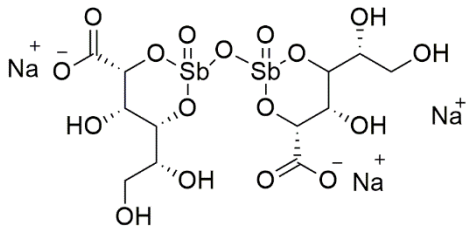
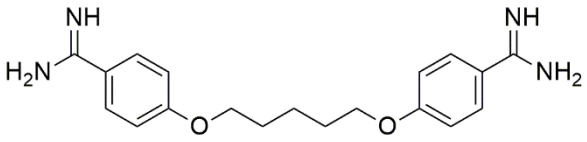


Sodium stibogluconate



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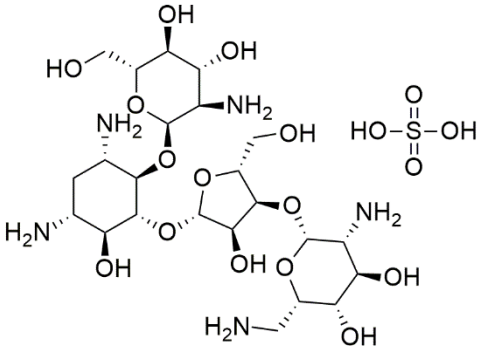
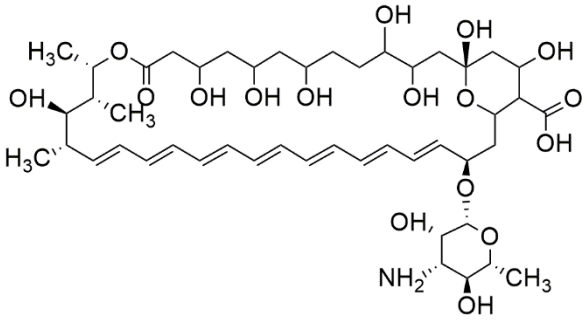
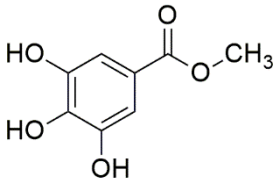


Paromomycin sulphate



Amphotericin B

Figure 1. Structures of some currently used antileishmanial drugs



Methyl gallate

Figure 2: Structure of methyl gallate

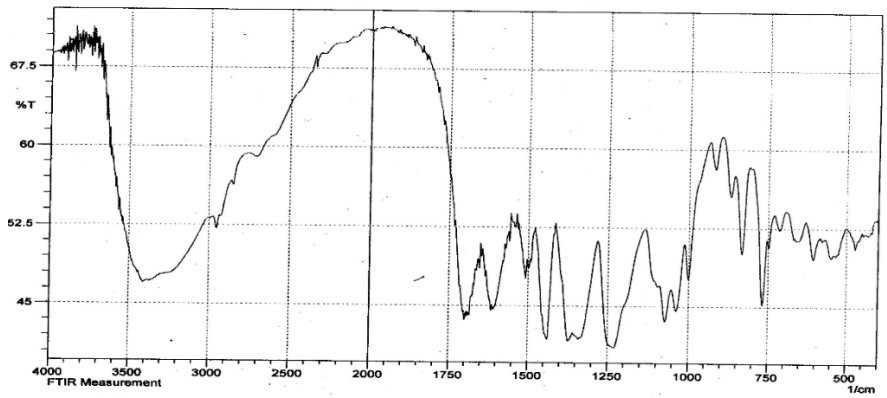


Figure 3: The IR spectrum of the isolate

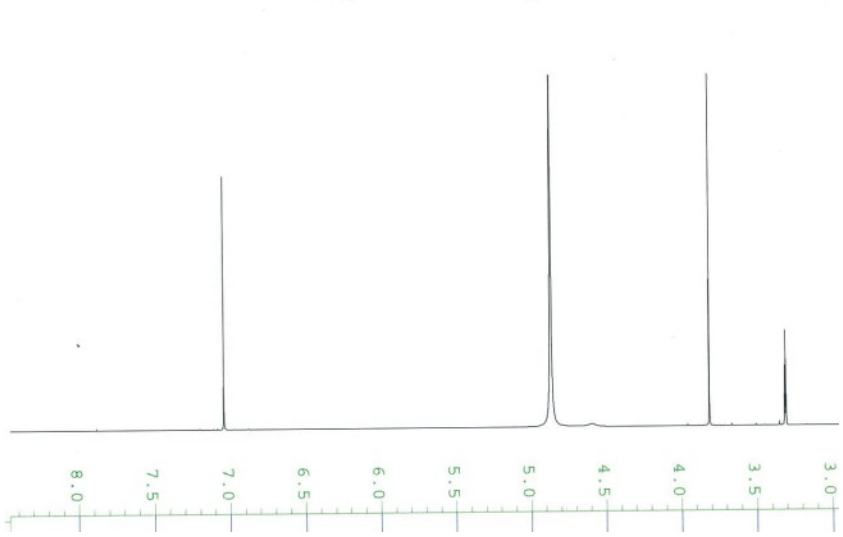


Figure 4: H1NMR spectrum of the isolate

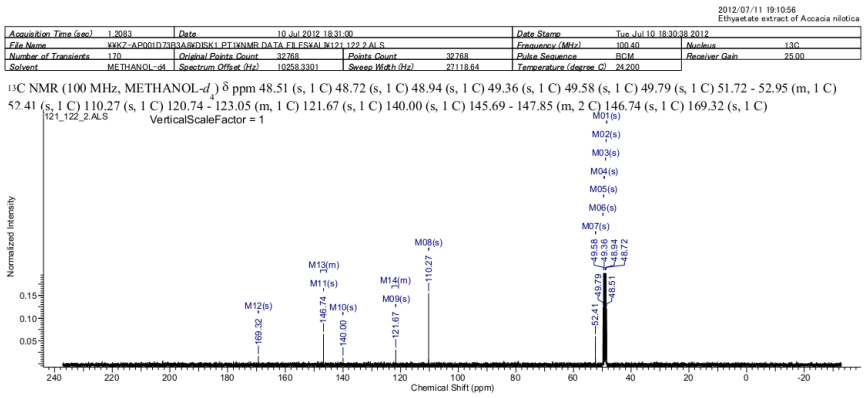


Figure 5: C13NMR of the isolate

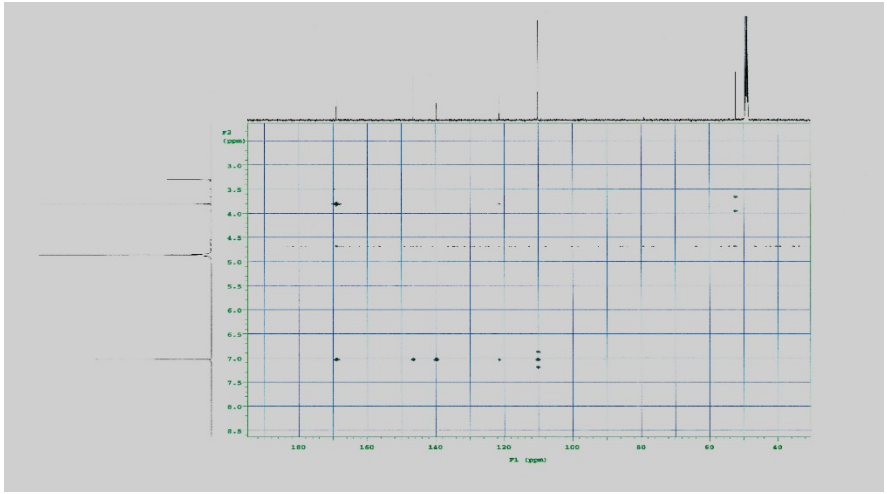


Figure 6: HMBC spectrum of the isolate

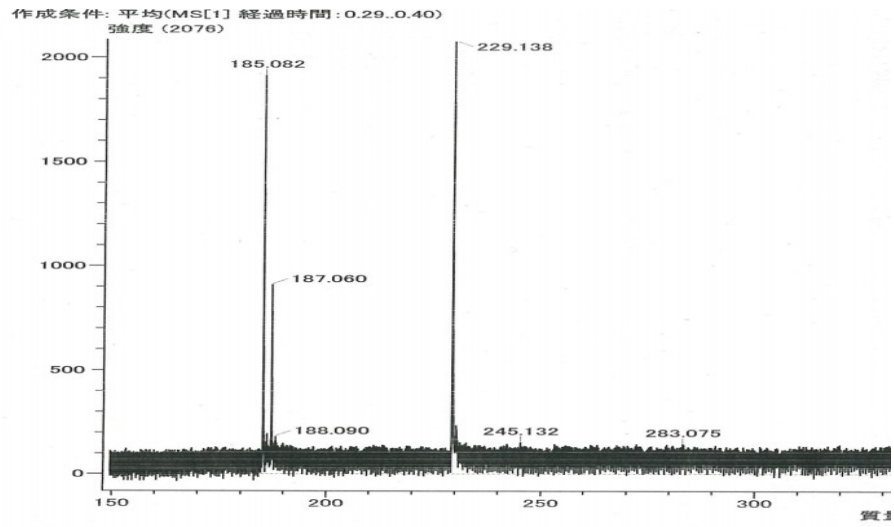


Figure 7: The ESI spectrum of the isolate