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Anti-Tubercular Activities and Molecular Characterization of Salivary Extract of Leech (*Hirudo medicinalis*) against *Mycobacterium tuberculosis*

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Abstract

Leech salivary extract (LSE) was screened for the anti-tubercular activity against *Mycobacterium tuberculosis* using microbroth dilution method. The bioactive components in LSE were determined using Shimadzu GC-MS QP2010 SE. The LSE exhibited bactericidal effects on *M. tuberculosis* at 50% (v/v). The result of the GC-MS analysis of LSE revealed seventeen (17) bioactive components. The major fatty acid components were oleic acid (33.9%) and palmitic acid also known as hexadecanoic acid (22.6%). Other bioactive components detected in appreciable amounts in LSE include, 4-bromobutyric acid (16.86%), 6,17-Octadiene-1-ol acetate (9.78%) and octahydro-1,4,9,9-tetramethyl (5.21%). The anti-tubercular activity of the LSE was attributed to these bioactive components. The results obtained suggest that leech salivary extract could be used in treating tuberculosis.

Keywords

GC-MS, *Mycobacterium tuberculosis*, Leech Salivary Extract

1. Introduction

Tuberculosis (TB) is a human global health disease primarily caused by *Mycobacterium tuberculosis* with other members of the *Mycobacterium* complex

(*Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canetti* and *Mycobacterium microti*) which have been implicated in pathogenesis [1]. The disease is complicated due to the emergence of multidrug resistant (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* [2]. Tuberculosis is one of the deadliest infectious diseases of humans [3]. Current TB treatment is a long course of combination of two or more antibiotics which is associated with toxic side effects and poor patient compliance [4] that has led to the recent report of drug resistant strains of *M. tuberculosis* [1]. The problem of drug resistance is coupled with mycobacterial persistence in mammalian cells, thus the need for the development of such drugs that will not only be active against the *Mycobacterium* spp but to as well kill the persistent *Mycobacterium* spp within the shortest possible time. With the urgent need for new anti-TB agents, it is particularly appropriate at this time to explore animal origin for the development of new anti-TB drugs. Thus leech salivary extract was selected for its anti-tubercular potentials in the present study.

Leech salivary extracts have received much attention because of their extensive uses in many medical fields. Leech saliva contains bioactive compounds that act as strong anticoagulants. They are mostly proteins and peptides secreted by the leech salivary glands while sucking the blood to prevent it from clotting. Hirudin is the most popular anticoagulant compound isolated from leech, *Hirudo medicinalis* [5]. Other species of leeches have been studied and a number of proteins and peptides have been found in their salivary extract [6]. Leeches have been used for treatment of a wide range of diseases such as skin diseases, nervous system abnormalities, urinary and reproductive system problems, inflammation, and dental problems and, also, the conventional use for bleeding [7].

2. Materials and Methods

2.1. Collection and Identification of Leech

Leeches were collected from a fresh water dam in Panda Development Area, Karu Local Government Area, Nasarawa State, Nigeria between September, 2014 and January, 2015 and were identified as *Hirudo medicinalis*, by a Zoologist in the Department of Biological Sciences, Federal University of Technology Minna, Niger State.

2.2. Laboratory Maintenance of Leeches

The leeches were maintained in well-aerated plastic containers filled with non-chlorinated water (borehole water), at room temperature ($28^{\circ}\text{C} \pm 2^{\circ}\text{C}$) throughout the period of study. The water was changed every three days and the leeches were fed with cow blood intervals of three weeks [8].

2.3. Extraction of Leech Saliva

Ice-shock method, as described by Abdulkader *et al.* [8] was employed for the extraction. This method involved putting the leeches in a well-closed glass test

tube immersed in an ice container. The set-up was then allowed to stand for 15 - 20 minutes. The leeches at this point were completely paralyzed and forced to regurgitate their intestinal content that appeared foamy. The salivary extract was then aspirated using a sterile hypodermic needle and syringe and then transferred into sterile container. Bloody salivary extract was discarded. The extract was then preserved at -4°C .

2.4. Anti-Tubercular Assay

2.4.1. Source of Microorganism

Clinical isolates of *Mycobacterium tuberculosis* was used for this study. The organism was obtained from Directly Observed Treatment Strategy (DOTS) Unit of the Diagnostic Laboratory of National Institute for Pharmaceutical Research and Development (NIPRD) Idu, Abuja, Nigeria. A total of three hundred (300) positive samples were obtained from TB patients attending the diagnostic Center.

2.4.2. Identification and Confirmation of Strain of *Mycobacterium tuberculosis*

The method described by NIPRD [9] was used for identification and confirmation of the organism. *Mycobacterium tuberculosis* stock solution was prepared by loop inoculation of 10ml sterile Middle Brook 7H9/Tween/ADC broth medium. The setup was then incubated at 30°C for 5 - 7 days. Confirmation and identification of the organism was done using the hot Ziehl-Neelsen staining technique.

2.4.3. Standardization of *Mycobacterium tuberculosis*

A measured quantity of 50 μl of *Mycobacterium tuberculosis* stock culture was inoculated into 50 ml of sterile 7H9/Tween/ADC broth medium. The setup was incubated at 30°C for 5 - 7 days. The turbidity of the culture was compared with 0.5 McFarland turbidity standards (approximately 1.5×10^7 CFU/ml). The culture was standardized to 10^6 cfu/ml. The standardized culture was then used for anti-tubercular screening [9].

2.4.4. Screening of LSE for Anti-Tubercular Activity

The anti-tubercular assay was carried out using the microbroth dilution method as modified by NIPRD [9]. The LSE was centrifuged at 3000 rpm for fifteen minutes to reduce particles that may interfere with the interpretation of the result. The filtrate was then used to screen for anti-tubercular activity. Fifty microliter (50 μl) of sterile 7H9/Tween/ADC broth was transferred into the micro well plate labeled 2 - 12, using a multichannel pipette. Fifty microliter (50 μl) of extract was then transferred into each of the 1st well. Fifty microliter (50 μl) of extract was also transferred into the 2nd well from which a double fold dilution was made by pipetting 50 μl of the mixture of extract and broth in well 2 into well 3 and mixed. Furthermore, 50 μl of the mixture in well 3 was transferred into well 4 and mixed. This was done until the 10th well. The 11th and 12th wells

were left without extract. 50 μ l of standardized *Mycobacterium tuberculosis* was inoculated each into wells 1 - 9 and well 11. Well 12 was left without culture to serve as medium sterility Control. Well 1 contained extract and culture, wells 2 - 9 contained extract, culture and broth, well 10 contained extract and broth to serve as extract sterility control, well 11 contained broth and culture to serve as organism viability control. The micro well was then incubated at 30°C for 5 - 7 days. The LSE efficacy was compared with Rifampicin (20 μ l/ml) which is a first line medication used in the treatment of tuberculosis. The experiment was done in triplicates. After the incubation period, 50 μ l of tetrazolium dye was added into each well (wells 1 - 12) and incubated for 20 minutes to check for color change. The wells with no color change were regarded as activity of extract indicating inhibition of *Mycobacterium tuberculosis*.

2.4.5. Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Minimum inhibitory concentration (MIC) of the LSE was determined using the microbroth dilution method of NIPRD [9]. The MIC of the extract was taken as the least concentration that inhibits the growth of *Mycobacterium tuberculosis*. The minimum bactericidal concentration (MBC) of the LSE was determined using the methods of NIPRD [9] that employed the prolonged incubation method. The test micro-titer plate was further incubated for 5 - 7 days. This was done to certify the LSE as bacteriostatic or bactericidal agent.

2.4.6. GC-MS Analysis

The characterization of the bioactive components in LSE was done using GC-MS QP2010 Plus (Shimadzu, Japan). The identification of the bioactive components in the LSE was carried out using a QP2010 gas chromatography with Thermal Desorption System, TD 20 coupled with Mass Spectroscopy (Shimadzu). The ionization voltage was 70 eV. Gas Chromatography was conducted in the temperature programming mode with a Restek column (0.25 mm, 60 m, XTI-5). The initial column temperature was 80°C for 1min, and then increased linearly at 70°C to 220°C, held for 3 minutes followed by linear increased temperature of 10°C to 290°C for 10 minutes. The temperature of the injection port was 290°C and the GC-MS interface was maintained at 290°C. The LSE was introduced via an all-glass injector working in the split mode, with helium carrier gas low rate of 1.2 ml·min⁻¹. The identification of compounds was accomplished by comparison of retention time and fragmentation pattern, as well as with mass spectra of the GC-MS.

2.4.7. Identification of Bioactive Components in LSE

Identification of the active components in the LSE was done by comparing their retention indices, peak area percentage and mass spectra fragmentation pattern with those stored in the data base of National Institute of Standards and Technology (NIST) and mass spectra from literature. The name, molecular weight, formula, structure and bioactivities of the compounds were ascertained.

3. Results

3.1. Identity of Clinical Isolates

Positive slides showed the presence of red rods on a blue background [9].

3.2. Anti-Tubercular Activity of Leech Salivary Extract against *Mycobacterium tuberculosis*

The results of the anti-tubercular activity of the LSE are showed on **Table 1**. The results revealed that there was activity at 50% concentration (obtained by diluting 50 µl of LSE and 50 µl of broth) but on further dilution, there was no activity. The MIC and MBC were at a 50% concentration. Rifampicin was most active on *M. tuberculosis* at 25% concentration.

3.3. GC-MS Analysis of LSE

GC-MS chromatogram of the LSE showed seventeen peaks which indicated the presence of seventeen bioactive components (**Figure 1**). The class of compounds present in the extract includes derivatives of benzene, alkene, fatty acid, saturated and unsaturated fatty acid, carboxylic acid, esters and aldehyde derivatives. The retention time (RT), peak area percentage, molecular weight and bioactivities of the LSE are shown in **Table 2**.

4. Discussion

Products of natural sources contain and produce a variety of bioactive substances that could be used in many fields of medicine [10]. Anti-tubercular therapy has been made difficult because of the increased occurrence of multi-drug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB). Strategies have been implemented to curb this menace but despite

Table 1. Anti-tubercular activity of leech salivary extract against *Mycobacterium tuberculosis*.

Concentration of LSE (%)	LSE	Rifampicin (µg/ml)
50	+*#	+
25	-	+
12.5	-	+
6.25	-	+
3.12	-	+
1.56	-	+
0.78	-	+
0.39	-	+
0.19	-	+

–: No Activity, *: Minimum inhibitory concentration; +: Activity#; #: Minimum bactericidal concentration; LSE: Leech Salivary Extract.

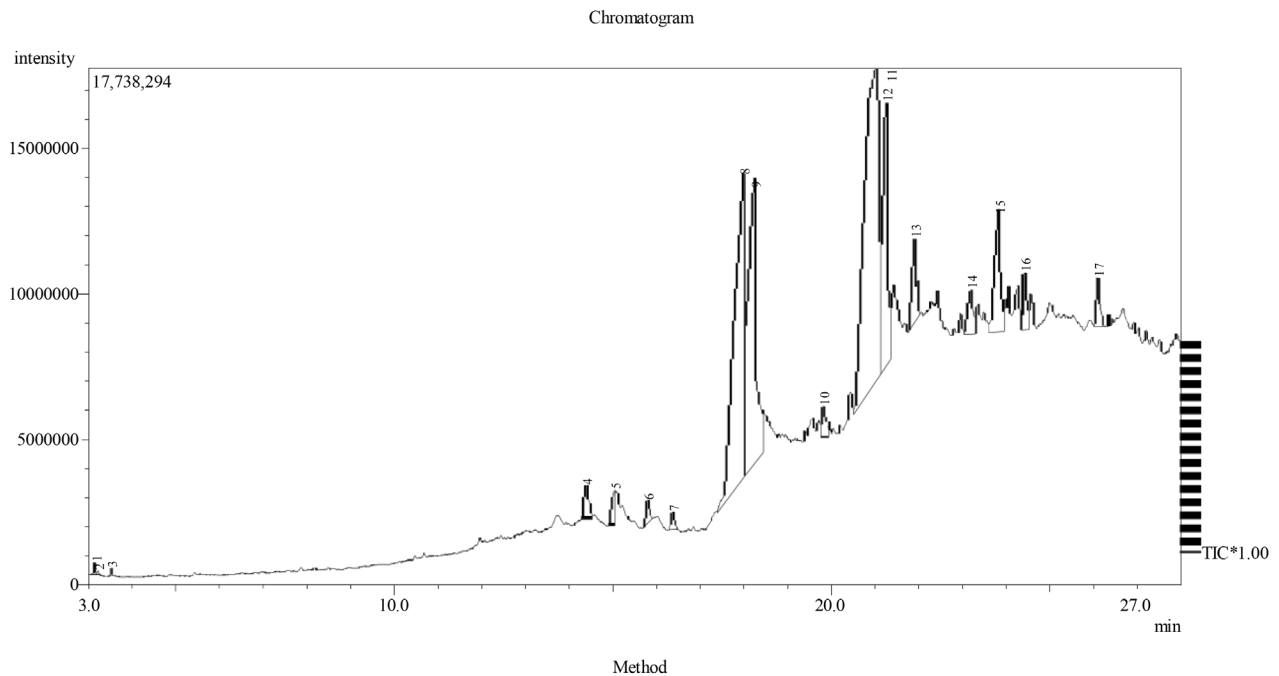


Figure 1. GC-MS chromatogram.

Table 2. GC-MS characterization of bioactive components of leech saliva extract.

S/N	RT (min)	PA (%)	MW (g/mol)	COMPOUND	BIOACTIVITY
1	3.149	0.13	153	Phosphonic acid	Antioxidant, anti-inflammatory, antimicrobial, anesthetic anti-candida and analgesic activity
2	18.248	16.86	166	4-bromobutyric acid	Antimicrobial
3	15.047	0.87	165	Methoxy-phenyl	Antimicrobial
4	21.030	1.13	141.06	2-aminoethyl hydrogen phosphate	Antimicrobial
5	23.831	5.21	206	Octahydro-1,4,9,9-tetramethyl	Antimicrobial
6	24.432	1.45	204	1,3,6,10 Dodecatetraene	Antimicrobial, antitumor, anti-inflammatory
7	26.114	1.29	186	Decanoic acid	Antimicrobial
8	3.233	0.05	284	Octadecanoic (Stearic) acid	Antimicrobial
9	18.005	22.69	256	Hexadecanoic (Palmitic) acid	Antimicrobial, antitubercular activity
10	16.378	0.47	266	Octadecenyl aldehyde	Antioxidant, anti-inflammatory, antimicrobial, anesthetic and analgesic activity
11	14.394	33.91	282	Oleic acid	Antimicrobial, anti-tubercular activity
12	15.808	0.58	270	Heptadecanoic (margaric) acid	Antioxidant, anti-inflammatory, antimicrobial, anesthetic and analgesic activity
13	3.530	0.08	342	Oxalic acid	Antimicrobial, antitumor, antimalarial
14	21.915	2.77	312	2,2 Dimethylpropionic acid	Antimicrobial, antitumor, antimalarial
15	21.272	9.78	308	6,17-Octadecadiene-1-ol acetate	Antimicrobial, antitumor, anti-inflammatory
16	19.823	0.96	282	n-Octadecanoic acid	Antimicrobial
17	23.199	1.78	298	9,12-Octadecadienoyl chloride	Antimicrobial, antitumor, anti-inflammatory

these strategies, the search for novel anti-TB drugs stimulates the investigation of natural products as an alternative regimen [11]. The LSE was tested *in vitro* for its activity against several clinical isolates of *M. tuberculosis*, using 7H9 Middlebrook broth containing ADC at different concentrations.

In the present study, the LSE exhibited pronounced bactericidal activity towards all the tested strains of *M. tuberculosis*, at 50% (v/v). This report is in agreement with the findings of Saludes *et al.* [12] in which *Morinda citrifolia* Linn. (Fruit) exhibited anti-tubercular activity against *M. tuberculosis*, as well as Soundhari and Rajarajan [13] in which *Allium cepa* and *Allium sativum* displayed anti-tubercular activity against *M. tuberculosis*. These authors opined that the activities of the extracts on *M. tuberculosis* could be due to the presence of certain chemical constituents of the extract, such as oleic acid and palmitic acid. Egharevba *et al.* [14] also, reported anti-tubercular activity of essential oil of orange (*Citrus sinensis*) peel at 25% (v/v) and seed oil of *Moringa oleifera* [15]. They reported that the anti-tubercular activity of the seed oil of *M. oleiferas* attributable to the oleic acid and palmitic acid content which amounted to about 94% of the oil fatty acid/derivatives composition. Rifampicin was more potent in the current investigation than LSE at a lower concentration possibly because it is a pure compound.

The result of GC-MS analysis of LSE revealed seventeen (17) components including two major fatty acids components. The most abundant component is oleic acid (33.9%) followed by palmitic acid also known as hexadecanoic acid (22.6%) amounting to 56.59% of the total composition of the bioactive components present in LSE. The percentage composition was determined using the % peak area of the constituents. These constituents are fatty acids of saturated and unsaturated derivatives. The anti-bacterial and antioxidant activity of fatty acids have been well documented [15] [16]. The biological activity of the LSE may, perhaps, be due to the presence of fatty acid components, especially the oleic and palmitic fatty acids. Santhosh and Suriyayanan [17], revealed that fatty acids such as oleic acid and linoleic acid exhibited activity against MDR forms of *M. tuberculosis*. In another study, Juan *et al.*, [18] reported the anti-tubercular activity of linoleic, oleic and palmitic acids found in essential oils from *Citrus* species. Sandoval-Montemayor *et al.* [19] also reported the anti-tubercular activity of commercially available palmitic acid and oleic acid against *M. tuberculosis* at MIC of 50 µg/ml and 100 µg/ml, respectively.

5. Conclusion

The study revealed that the LSE is rich in oleic acid (33.9%) and palmitic acid (22.6%). The LSE was found to be active against *Mycobacterium tuberculosis* at 50% (v/v). The anti-tubercular activity of the LSE was attributed to the high oleic and palmitic acid contents since both compounds had been previously reported to possess anti-tubercular activities. The findings in the study could be exploited in new anti-tubercular drug research and drug design.

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