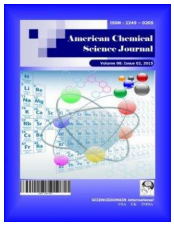

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Identification of Thymol as an Antitubercular Agent from *Ocimum gratissimum* Leaf Essential Oil

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SEO and JIO designed the study, performed hydrodistilation, fractionation and isolation of thymol, wrote the protocol, and wrote the first draft of the manuscript. Author PO performed the biological evaluation. Author ATO obtained the NMR and GCMS spectra and performed compound library searches. Author SEO managed the literature searches and analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Ocimum gratissimum consumed as a vegetable is used to treat cough, tuberculosis and dysentery in Nigeria. Hydro-distillation of the fresh leaves of *O. gratissimum* yielded a golden yellow essential oil 0.5% v/w which exhibited minimum inhibitory concentration (MIC) of 48 µg/ml against *Mycobacterium bovis* BCG strain. Activity-guided fractionation of the oil into neutral and acidic portions followed by column chromatography led to the isolation of a crystalline compound with MIC

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of 9.8 µg/ml while the control drug isoniazid had MIC of 0.07 µg/ml. GC-MS and ^{1}H NMR analyses revealed the bioactive crystalline compound to be thymol.

Keywords: Ocimum gratissimum; essential oil; acidic fraction; thymol; antitubercular activity.

1. INTRODUCTION

Tuberculosis (TB) continues to be a major public health threat. Tuberculosis (TB) remains one of the world's deadliest communicable diseases. In 2013, an estimated 9.0 million people developed TB and 1.5 million died from the disease. Nigeria is one of the high burdened TB countries in the world [1]. Current drugs include rifampicin, isoniazid, streptomycin, ethambutol and pyrazinamide. These drugs were developed over 50 years ago and the problem of resistance and low therapeutic efficacy are common with these medications. Resistance, long duration of therapy, side effects of current drugs and co-infection with HIV have led to the search for new drugs. Over 80% of the world population uses traditional medicines for the treatment of different diseases and ailments including tuberculosis [2]. With drugs such as paclitaxel and artemisinin among others having their origin from plants, we are optimistic that safe and efficacious drugs for the management of tuberculosis could be obtained from plant sources.

Ocimum gratissimum L. belongs to the lamiaceae family. It is a vegetable commonly known as scent leaf in Nigeria. It is known amongst the Ibos as nchuanwu; the Yorubas as efirin; the Hausas as daddoyar gida and the Edos as alumọkhọ. *O. gratissimum* is a shrub that grows 1-2 m tall, is anthropogenic of village areas and not found in the wild. In Nigeria the leaf serves as a remedy for bronchitis, sinusitis, cough, tuberculosis, headaches, and as a decongestant for head-colds. In Gabon *O. gratissimum* leaf infusion is given for chesty condition and in Uganda the leaf crushed in cold water is taken for cough [3,4]. The essential oil of *Ocimum gratissimum*, growing wild in Rwanda analysed by GCMS gave 35% thymol and 11% eugenol [5]. Eugenol was identified as the antibacterial active compound in *Ocimum gratissimum* essential oil [6].

Thymol had been identified as one of the major constituents of *Ocimum gratissimum* essential oil [7,8]. The aim of this study was to identify the antitubercular active compound in the fresh leaf essential oil of *O. gratissimum.*

2. MATERIALS AND METHODS

Petroleum ether (40-60), hexane, DMSO and ethyl acetate were Analar grade from BDH Chemical Ltd (Poole, England), alumina (type H) was from Merck (Darmstadt, Germany). Isoniazid analytical standard ≥ 99% was from Fluka (Germany), Middlebrook 7H9 Broth (Sigma Aldrich).

GCMS and NMR facility was at the National Institute of Allergy and Infectious Diseases, National Institute of Health, USA.

2.1 Plant Material

Fresh *Ocimum gratissimum* were collected from Idu, Abuja, Nigeria and was identified and authenticated at the herbarium of the National Institute for Pharmaceutical Research and Development, Idu Industrial Area, Abuja, Nigeria where voucher specimen 5942 was deposited.

2.2 Extraction and Isolation

Hydro-distillation of the fresh leaves of *O. gratissimum* (1 kg) for 4 hours yielded 5ml (0.5% v/w) of golden yellow essential oil [9]. The essential oil (2 g) was dissolved in 50 ml of hexane and extracted with 1 M NaOH (2 x 30 ml) using separating funnel [10]. The aqueous portions were combined and washed with hexane. All the hexane portions were combined, dried with anhydrous sodium sulphate and concentrated to dryness using rotary evaporator to obtain the neutral fraction (NF). The combined aqueous portion was acidified with concentrated hydrochloric acid drop-wise until oily layer forms above the aqueous layer. The mixture was frozen, thawed and filtered within 10 minutes. The residue was collected on the filter paper and dried in a desiccator to obtain the acidic fraction (AF).

2.2.1 Column chromatography of acidic fraction

The acidic fraction (0.5 g) was subjected to open column chromatography on alumina using a step gradient of petroleum ether and diethyl ether

100:0 (100 ML), 95:5 (100 ML) and 90:10 (500 ML). A total of seven fractions (1-7) x100 ML were obtained. The fractions were monitored on TLC [10]. Fractions 3-6 gave a single spot on TLC and showed enhanced antitubercular activity.

2.2.2 Recrystallization of bioactive fraction

Fractions 3-6 were combined and recrystallization from petroleum ether gave colourless crystalline compound (240 mg, 19% w/w), which was submitted for GCMS, NMR analyses and biological evaluation against *M. bovis*.

2.3 Gas Chromatography-Mass Spectrometry Analysis

The GC-MS analysis was carried out on a Hewlett Packard 5890 gas chromatograph equipped with FID detector and coupled with a mass spectrometer of the Hewlett Packard 5971 type operating in the EI mode at 70 eV. Capillary column DB-5 (30 m x 0.25 mm i.d.; film thickness 0.25 µm) was used. DB-5 column operating conditions were as follows: from 50ºC (5 min), 50ºC to 250ºC at the rate of 2ºC/min. The injector and detector temperatures were 250ºC and 300ºC, respectively. The carrier gas was helium at a flow rate of 1.50 ml/min. Sample in hexane (0.2 µl) was injected [11].

2.4 ^{1}H NMR Analysis

^{1}H NMR data were obtained using Varian Gemini 300 MHz [12]. The compound was dissolved in deuterated chloroform ($CDCl_3$).

2.5 Antimycobacterial Assay

Determination of antitubercular activity was carried out on *Mycobacterium bovis* BCG strain (ATCC 35737) using the broth dilution method previously described [4,13,14]. 100 mg each of the sample was dissolved separately in 1 ml of dimethylsulphoxide (DMSO). The solutions were centrifuged for 20 minutes at 13,000 rpm. A ten-fold dilution of the each solution in 7H9 Middle Brook broth was made, to give a final concentration of 10 mg/ml solution. 50 µl of media was introduced into all wells 2 to 12 of a 96-well micro-titer plate, while 100 µl each of the sample diluted in 7H9 Middle Brook broth was delivered into the appropriate well 1 of the 96-well plate. Two-fold dilutions were performed by sequential transfer of 50 µl of each sample from well 1 to 2; after thorough mixing, 50 µl was transferred from well 2 to 3. The process was repeated through to well 11 where 50 µl was discarded leaving column 12 for the negative control. 50 µl of inoculum prepared by diluting a 5 -7 day old culture of *Mycobacterium bovis* BCG (OD 0.2-0.3) 1:1000 (by adding 50 µl of cell culture into 50 ml 7H9/ADC medium) was added to all the wells and incubated for 14 days at 37^{o}C, after which the growth or inhibition of growth was read by direct recording of visual growth (Fig. 1). All of the MIC determinations were done in duplicate. The MIC was reported as the highest concentration of sample resulting in complete inhibition of visual growth. DMSO was used as negative control and isoniazid as positive control.

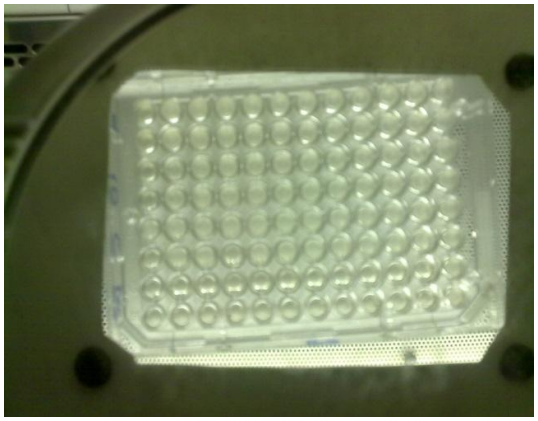

Fig. 1. 96-well microliter plate for MIC determination

3. RESULTS AND DISCUSSION

Hydro-distillation of the fresh leaves of *O. gratissimum* (1 kg) for 4 hours yielded a golden yellow essential oil 5 ml (0.5% v/w) which exhibited minimum inhibitory concentration of 48 µg/ml against *M. bovis* BCG.

The essential oil (2 g) dissolved in hexane and extracted with 1 M NaOH (2 x 30 ml) using separating funnel and worked-up yielded neutral fraction (0.65 g, 32.5%) and acidic fraction (0.8 g, 40%).

Antitubercular screening of the neutral fraction and acidic fraction showed the acidic fraction to be most active with MIC of 12.6 µg/ml, compared with the neutral fraction with MIC of 63 µg/ml. Column chromatography of the acidic fraction

(0.5 g) on alumina using a step gradient of petroleum ether and diethyl ether yielded a total of seven fractions of which fractions 3-6 gave a single spot on TLC and showed enhanced antitubercular activity and recrystallization from petroleum ether gave colourless crystalline compound (240 mg, 19% w/w) with MIC of 9.8 µg/ml. The control drug isoniazid had MIC of 0.07 µg/ml. Previous antitubercular screening of the of the water extract of *O. gratissimum* against *M. bovis* BCG gave MIC value greater than 2500 µg/ml [12]. This weak antitubercular activity observed for the water extract compared to the essential oil could be attributable to the fact that thymol is only very slightly soluble in water, but very soluble in oils and 1 M NaOH [15].

The gas chromatograph of the compound gave a single peak that eluted at 13.4 minutes. The mass spectra of the compound gave m/z 135 (100%), 115 (18%), and 150 (35%) as the molecular ion which is characteristic of thymol (Fig. 2).

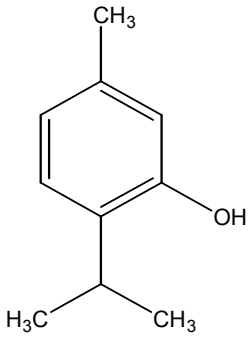

Fig. 2. Thymol

The ^{1}H NMR spectra (Fig. 3) of the compound showed chemical shift (δ) absorptions at 1.17 (6 H, d, J=6.5 Hz), 3.2 (1 H, m), 2.25 (3 H, s), 6.75 (1 H, s), 6.85 (1 H, d, J=7.8) and 7.15

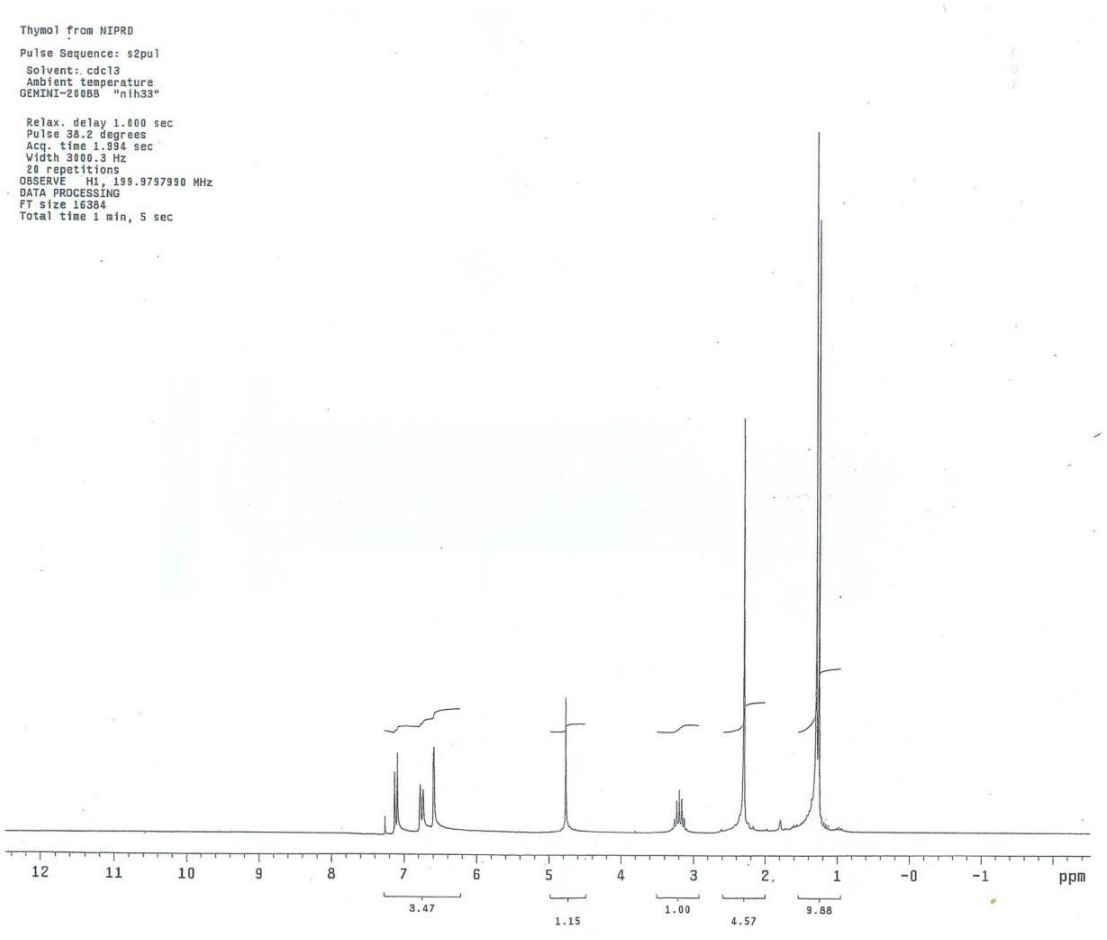

Fig. 3. ^{1}H NMR spectrum of thymol

(1 H, d, J=7.8) consistent with published data [16] for thymol (Fig. 2). The compound had melting point of 49.5ºC. The melting point remained undepressed when mixed with commercial authentic thymol.

4. CONCLUSION

The present study is the first report of thymol being the antimycobacterial agent in *Ocimum gratissimum* leaf. This provides justification for the traditional uses of *Ocimum gratissimum* leaf for the management of cough and tuberculosis in Nigeria. Hydro-distillation of the fresh leaves of *O. gratissimum* gave a golden yellow essential oil in 0.5% v/w yield which exhibited MIC of 48 µg/ml against *Mycobacterium bovis* BCG. Activity-guided fractionation of the oil led to the isolation of thymol from the acidic fraction with MIC of 9.8 µg/ml.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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