NISEB JOURNAL Vol. 11, No. 4, October 31, 2011
Printed in Nigeria

1595-6938/2011 $5.00 + 0.00

http://www.nisebjournal.org

NISEB 2011112/11409

Antimicrobial properties of metabolites produced by *Klebsiella aerogenes* and *Bacillus pumilus*

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(Received May 20, 2011; Accepted July 8, 2011)

ABSTRACT: Natural organic compounds produced by microorganisms are important screening target for a variety of bioactive substances. Some bacteria which are normally susceptible are daily reverting to resistant forms. On this premise, the search for new and potent antimicrobial substances especially, those that will be able to check the development and/or resurgence of resistant strains is the basis for this work. The aim of this study was to isolate and characterize those organisms that can produce antimicrobial metabolites of fatty acid origin, its effect, and investigate the bacteriocidal activities of the metabolites by determining the rate of killing of the test agents. Metabolites obtained from *Klebsiella aerogenes* (CWS) and *Bacillus pumilus* (IWS) were found to exhibit anti-microbial properties against a wide range of organisms. The metabolites, CWS and IWS, resulted in diameters of zone of inhibition of 13 and 17 mm when tested against *Pseudomonas aeruginosa,* and 17 and 18 mm when tested against *Bacillus subtilis* respectively. The bacteriocidal activity showed that a concentration of 0.15 mg ml^{-1} of *Klebsiella aerogenes* metabolites reduced the population of *Bacillus subtilis* from 2.4 x 10^7 to 1.3 x 10^7 cfu ml^{-1} at 10 min incubation period. There was no significant difference in the population density of *Bacillus subtilis* after 10 min exposure to 0.15 mg ml^{-1} of *Bacillus pumilus* metabolites (4.0 x 10^7 to 3.5 x 10^7 cfu ml^{-1}). A similar trend was obtained for *Pseudomonas aeruginosa* with both metabolites. A low minimum inhibitory concentration (MIC) of 0.05 mg ml^{-1} for (CWS) and 0.05 and 5.0 mg ml^{-1} for (IWS) in *Pseudomonas aeruginosa* and *Bacillus subtilis* were obtained respectively. The infra-red spectral analysis (IR) of the metabolites revealed several functional groups like carboxylic acid, aldehyde and ester carbonyl. A component of each of the metabolites corresponded to 10-hydroxyoctadecanoic acid R_f value of 0.35. It can be concluded that the metabolites produced by *Klebsiella aerogenes* and *Bacillus pumilus* can serve as effective antimicrobial agents.

Key words: Metabolites, Anti-microbial, bacteriocidal, 10-hydroxyoctadecanoic acid.

Introduction

For a very long time in history different metabolites have been screened for their biological effects including antimicrobial activities. Natural organic compounds produced by microorganisms are important screening target for a variety of bioactive substances (Chhiaki *et al.,* 2007). However, the rate of discovery of novel substances from microorganisms, especially from actinomycetes of terrestrial origin has recently decreased (Takizawa *et al*., 1993).

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Many microorganisms are increasingly becoming resistant at a fast rate to many of the drugs currently used in medical practice (Pihlajamaki *et al*., 2002; Rantala *et al*., 2005). Some - Gram-negative bacteria (e.g. *Pseudomonas, Proteus,* and *Salmonella*) have been known to be resistant to some commonly used drugs and members of the genus *Staphylococcus,* which are normally susceptible are daily reverting to resistant forms (Oloke, 1989). The relevance of information obtained on the resistance of bacteria to antibiotics is to appreciate the magnitude of the problems and establish baselines for taking action (Caprioli *et al.*, 2000). On this premise, the search for new and potent antimicrobial substances especially, those that will be able to check the development and/or resurgence of resistant strains or forms of organism under control is the basis for this work. The aim of this study was to isolate and characterize those organisms that can produce antimicrobial metabolites of fatty acid origin,its effect and investigate the bacteriocidal activities of the metabolites by determining the rate of killing of the test agents.

Materials and Methods

Sample source

The leaf samples used include fresh leaves of *Lantana camara L*. (verbenaceae) – Lantana (Ewon-agogo), *Hibiscus Sabdariffa L*. (Malvaceae) – Rosella (Isapa) and *Bougainvillea spectabilis* all from the University of Lagos, Akoka-Yaba, Lagos (Table 1). Soil and water samples used were obtained from the University campus and Ijeshatedo community in Aguda, Lagos.

Table 1 Sample names with their corresponding codes

Samples	**Code**
Lantana camara	LC
Hibiscus sabdariffe	HS
Bougainvillea spectabilis	BS
Ijesha water sample	IWS
Ijesha soil sample	ISS
Campus water samples	CWS
Campus soil sample	CSS

Test organisms

The test organisms used for screening for the antibacterial activity were obtained from the University Teaching Hospital, Idi-Araba, Lagos. They include *Pseudomonas* spp, *Proteus* spp, *Klebsiella* spp, *Lactobacillus* spp1, *Candida albicans, Salmonella* spp, *Bacillus subtilis, Staphylococcus aureus,* and *Lactobacillus* spp2. The microorganisms were subcultured on yeast extract agar slants.

Isolation of microorganisms and inoculation of culture media

Soil Samples (10g) were weighed into 90 ml of sterile distilled water in a test tube and shook vigorously. The soil particles were allowed to settle. One ml of supernatant was transferred to a special broth medium containing $MgSO_4$ (0.2g), KH_2PO_4 (1 g) and groundnut oil (1 ml) in 100 ml of sterile distilled water. This was allowed to stand for three days with intermittent shaken. It was then streaked unto a nitrogen-free agar medium containing the following composition: glucose (4%), KH_2PO_4 (0.1%), $MgSO_4$ (0.02%) and agar powder (1% w v^{-1}) . The isolates

obtained were subcultured using yeast extract agar (2.3g 100 ml^{-1}) at 37^{o}C for 18-24 h. Pure isolates were maintained on yeast extract agar. The isolates were resuspended in yeast extract broth for subsequent analysis. The procedure was also repeated for the water samples (10 ml in 90 ml sterile distilled water). Leaves were placed in 10 ml of sterile distilled water containing 0.1g of KH_2PO_4. After 2 to 3 days, 1 ml of the solution was streaked on a nitrogen-free medium.

The isolates obtained were inoculated into 100 ml of SMD medium composed per litre of glucose, 10 g; KH_2PO_4, 5 g; soy bean meal, 5 g; yeast extract, 5 g; $FeSO_4.7H_2O$, 0.5 g; $MnSO_4.H_2O$, 0.008 g; $ZnSO_4$, 0.014 g and nicotinic acid, 0.01 g (Difco manual, 1984; Yorimitsu *et al.,* 2005). The medium was adjusted to pH 7.0 with dilute hydrochloric acid before seeding with isolates and incubated at 30^{o}C for 24 h. After 24 h of growth on SMD medium, 5 g of sterile lactose was added to each 100 ml SMD Medium cultured bottle. This was incubated for another 48 h and thereafter 3 ml of sterile groundnut oil was then added to the culture medium. It was incubated for 72 h at 37^{o}C with intermittent shaking, thereafter, the culture broth was acidified to pH 2.0 with 6N hydrochloric acid. The ether portion containing the metabolites was left in an open beaker to evaporate. After evaporation it was kept in a MacCarthney bottles which was preserved in the freezer for further analysis (Hou *et al.,* 1992).

Determination of MIC.

The value of the bacteriostatic (growth inhibition) activity of the metabolites were quantitatively expressed as the Minimum Inhibitory Concentration (MIC) which were determined using the paper disc method. An 18 h broth culture of each bacterium was used to seed solidified yeast extract agar plate. Paper discs of similar diameters (7 mm) were obtained by punch perforation of filter papers and oven sterilized at 160^{o}C for 1 h. The sterile discs were dipped into a tween-80 solution of each metabolite using sterile forceps and aseptically layered onto the inoculated yeast extract agar. Paper discs dipped in tween-80 and tetracycline solution of known concentrations served as negative and positive controls. Zones of inhibitions (in mm) were measured after 24 h of incubation.

Determination of microcidal rate

The bacteriocidal activities of the metabolites on the test microorganisms were demonstrated by determining the viability of the bacterial cells by means of viable counts using the surface plating techniques as described by Kolawole (1985). A 3 ml volume of each metabolite solution containing 0.05 mg ml^{-1} of the metabolites in tween-80 was mixed with 3 ml of a 24 h broth cultures of the isolates. They acted as reactant mixtures or stocks for the analysis. The suspension was held at room temperature and mixed thoroughly for 3 min. Samples (0.5 ml) were transferred at pre-determined intervals of time (0,10, 20, 30 and 60 mins) into 4.5 ml of recovery medium in test tubes, shaken thoroughly and diluted serially before plating out by spreading 0.2 ml on yeast extract agar. The plates were incubated at 37^{o}C for 18-24 h. Controls for these experiments consisted of 3 ml of organism suspension mixed in 3 ml of 50% tween-80.

Chromatographic separation

Crude extracts of the metabolites containing reaction products were subjected to Thin Layer chromatography (TLC) to separate the components. Thin Layer Chromatography (TLC) was carried out on 0.25 mm layers of silica gel GF254 (Marck) prepared on glass plates (5 by 20 cm or 20 by 20 cm) with a Quick fit industries spreaders. The purity of each spot was ascertained by different ratios of petroleum ether to diethyl ether and glacial acetic acid (90:10:1) as developing solvents. This was observed under UV light. The locating reagents was a solution of 0.1% 2,7-dichlorofluorescence in methanol. The plates were sprayed with 60 ml of methanol and 0.5 ml of H_2SO_4 with a spray gun, after warming to develop a brown colour.

Infrared (IR) Analysis

The Bulk Scientific IR (Model M500) transparent windows (Nacl disc) was used as sample holder. The two metabolites with better antimicrobial property (IWS and CWS) were smeared on the holder and scanned on the IR machine . The presence of any functional group in the metabolic structure was confirmed by IR spectral analysis in an intense absorption band at a specific wave number (cm^{-1}).

Results

The result of the zone of inhibition of metabolites against test organisms showed that the metabolites produced by *Klebsiella aerogenes* and *Bacillus pumilus* were active against almost all the test organisms. In Table 2, zones of inhibition of 10 and 7 mm were observed for the two metabolites respectively, when tested against *Staphylococcus aureus.* The diameters obtained when the metabolites were tested against *Pseudomonas aeruginosa* were 13 and 17 mm while that of *Bacillus subtilis* were 17 and 18 mm respectively. The metabolites produced by *Norcadia madunae* was less active than the metabolites produced by both *Klebsiella aerogenes* and *Bacillus pumilus.* Metabolites were prepared from seven organisms. However, the metabolites from *Klebsiella aerogenes* and *Bacillus pumilus* exhibited more antimicrobial activities. Also, when the metabolite of *Norcadia madunae* was tested against *Candida albicans* and *Bacillus subtilis*, the diameters of the zone of inhibition obtained were 11 and 14 mm respectively while the values obtained for the metabolites produced by *Bacillus pumillus* when tested against the pathogenic organisms were 12 and 18 mm respectively.

Table 2: Zone of inhibition of metabolites against test organisms

Name of organism	IWS	CWS	CSS	LC	BS	T-80
	Diameter of zones of inhibition (mm)*					
Staphylococcus aureus	7	10	6	IA	IA	IA
Pseudomonas aeruginosa	17	13	IA	7	9	IA
Lactobacillus plantarum	5	6	7	6	IA	IA
Lactobacillus spp2 *(2nd strain)*	IA	12	11	IA	IA	IA
Klebsiella spp	IA	9	7	6	IA	IA
Proteus spp	7	8	7	8	10	IA
Bacillus subtilis	18	17	IA	14	7	IA
Salmonella spp	16	8	8	IA	12	IA
Candida albicans	12	IA	9	11	IA	IA

IWS, 50%v/v of metabolite produced by *B. pumilus*; CWS, 50% v/v of metabolite produced by *K. aerogenes*; LC, 50% v/v of *N. madunae*; CSS, 50% v/v metabolite from Campus soil sample; BS, 5-% v/v of metabolite from Bougainvillea spectabilis; T-80, Tween-80 used as solvent; IA, Inactive. *Each value is mean of three replicates.

The MIC's of the metabolite against *Salmonella* spp are 0.5mg/ml and 5.0mg/ml while against *Candida albicans* are 50 mg/ml and 0.5mg/ml respectively (Table 3).
The MIC's of *Bacillus pumillus* metabolite were higher and therefore less active than those of the metabolite produced by *Klebsiella aerogenes.* Generally, the two metabolites showed active antimicrobial properties (Table 3).

Table 3: Minimum Inhibitory Concentration (MIC) Produced by metabolites on tested isolates

Name of organism	IWS	CWS	DMSO
	Minimum Inhibitory Concentration (mg/ml)*		
Pseudomonas aeruginosa	0.05	0.05	IA
Lactobacillus spp2 *(2nd strain)*	0.05	5.0	IA
Bacillus subtilis	5.0	0.05	IA
Salmonella spp	0.5	5.0	IA
Candida albicans	50	0.5	IA

IWS, metabolite by *Bacillus pumilus*; CWS, metabolite by *Klebsiella aerogenes*; DMSO, (Dimethyl sulfur oxide), vehicle control; IA, Inactive; *Each value is an average of two readings.

Bacteriocidal activities of the metabolites

The bacteriocidal activity showed that a concentration of 0.15 mg ml^{-1} of the *Klebsiella aerogenes* metabolites reduced the population of *Bacillus subtilis* from 2.4 x 10^7 to 1.3 x 10^7 cfu ml^{-1} at 10 min incubation period. There was no significant difference in the population density of *Bacillus subtilis* after 10 min exposure to 0.15 mg ml^{-1} of *Bacillus pumilus* metabolites (4.0 x 10^7 to 3.5 x 10^7 cfu ml^{-1}). A similar trend was obtained for *Pseudomonas aeruginosa* with both metabolites. The cidal action of the metabolites on both *Bacillus subtilis* and *Pseudomonas aeruginosa* was achieved after 60 min of exposure to 0.15 mg ml^{-1} of the metabolite (Figs. 1 and 2). At a concentration of 0.15 mg ml^{-1}, the metabolite produced by *Bacillus pumilus* inhibited the growth of 1.0 x 10^7 cells ml^{-1} of *Bacillus subtilis* after 1 h of
interaction. Also, the same applies to the metabolite produced by *Klebsiella aerogenes* (Figs.1 and 2).

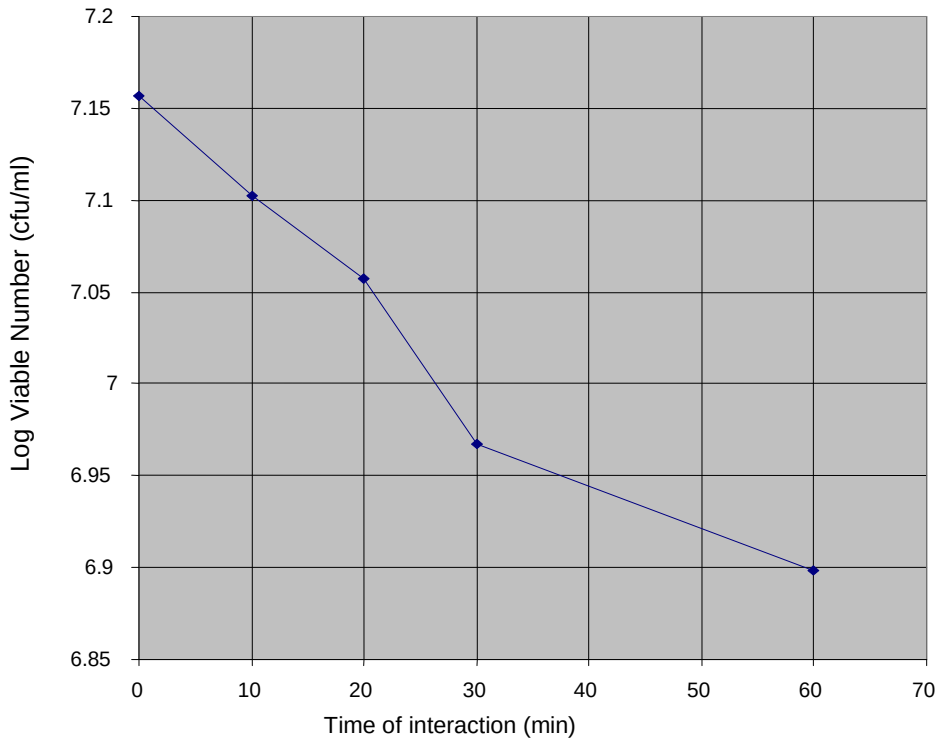


Fig. 1 Cidal activity of *Pseudomonas aeruginosa* by 0.15 mg ml^{-1} of IWS metabolite
Each point represents the average of three determinations

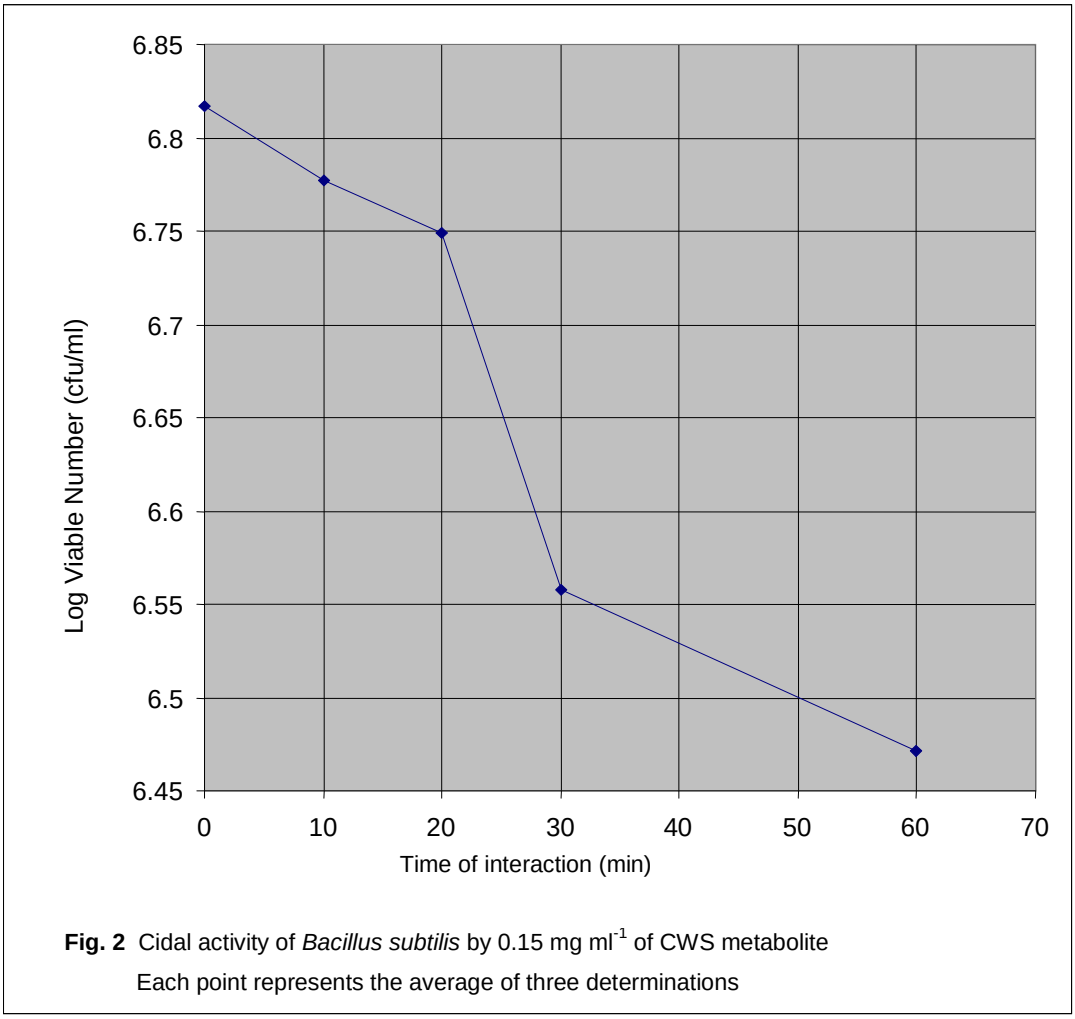


Fig. 2 Cidal activity of *Bacillus subtilis* by 0.15 mg ml^{-1} of CWS metabolite
Each point represents the average of three determinations

The infrared (IR) spectral analyses of the metabolites produced by *Klebsiella aerogenes* and *Bacillus pumilus* both have close ranges of absorptions. The intense absorption band at 1745.7 cm^{-1} (Figs. 3 and 4) showed the presence of an ester carbonyl in both samples; IWS and CWS metabolites. Absorptions at both 2858.3-2931.3 and 3363 cm^{-1} produced aldehyde and carboxylic acids respectively. Thus confirming strong suspicion of 10-hydroxyoctadecaenoic acid.

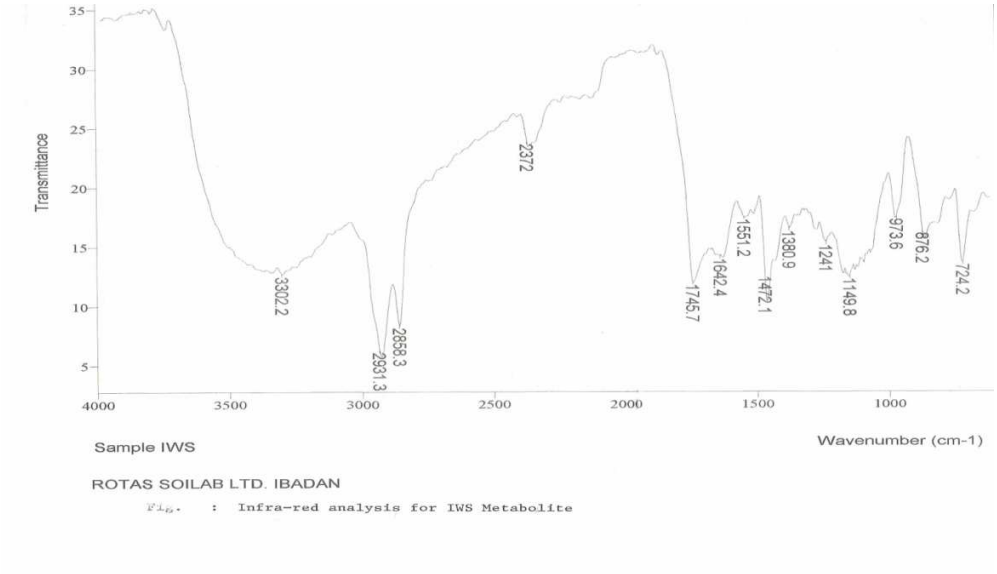


Fig. 3: Infrared analysis for IWS metabolite

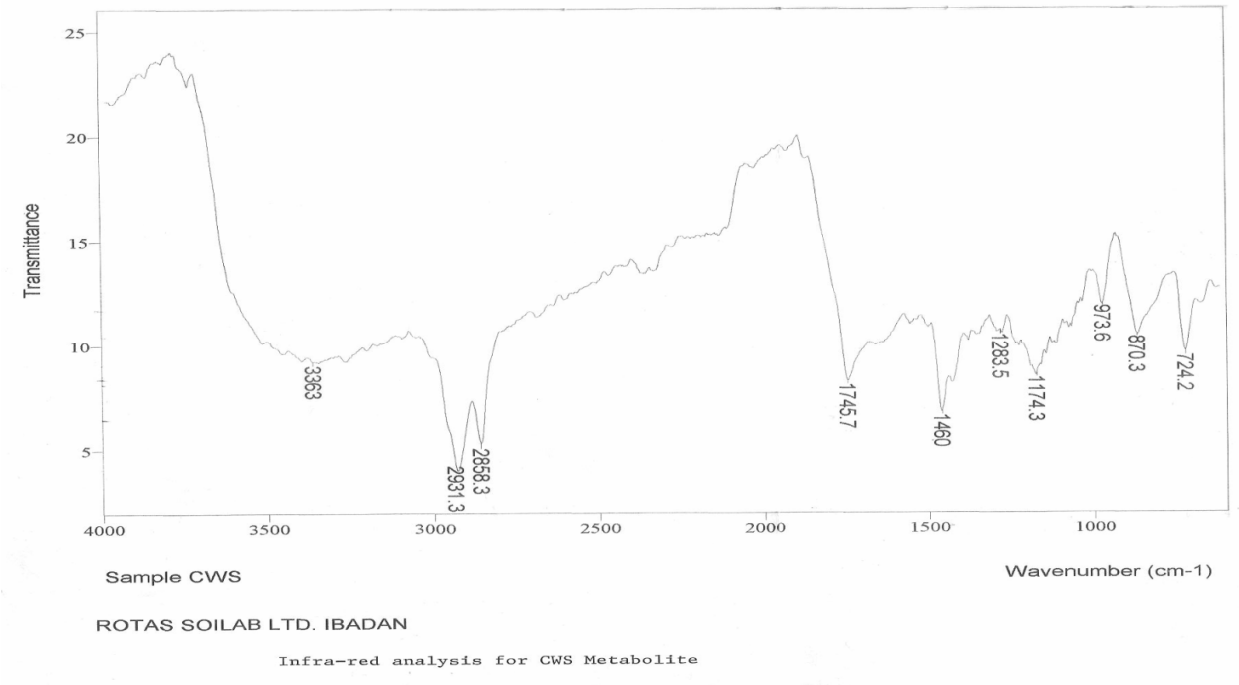


Fig. 4: Infrared analysis for CWS metabolite

The result of the thin layer chromatography analysis showed that the metabolites are made up of different components with the following R_f values: 0.69 for metabolite produced by *Norcadia madunae*; 0.35, 0.45 for *Klebsiella aerogenes* metabolite, and 0.35, 0.75 for *Bacillus pumilus* metabolite respectively (Figure 5).

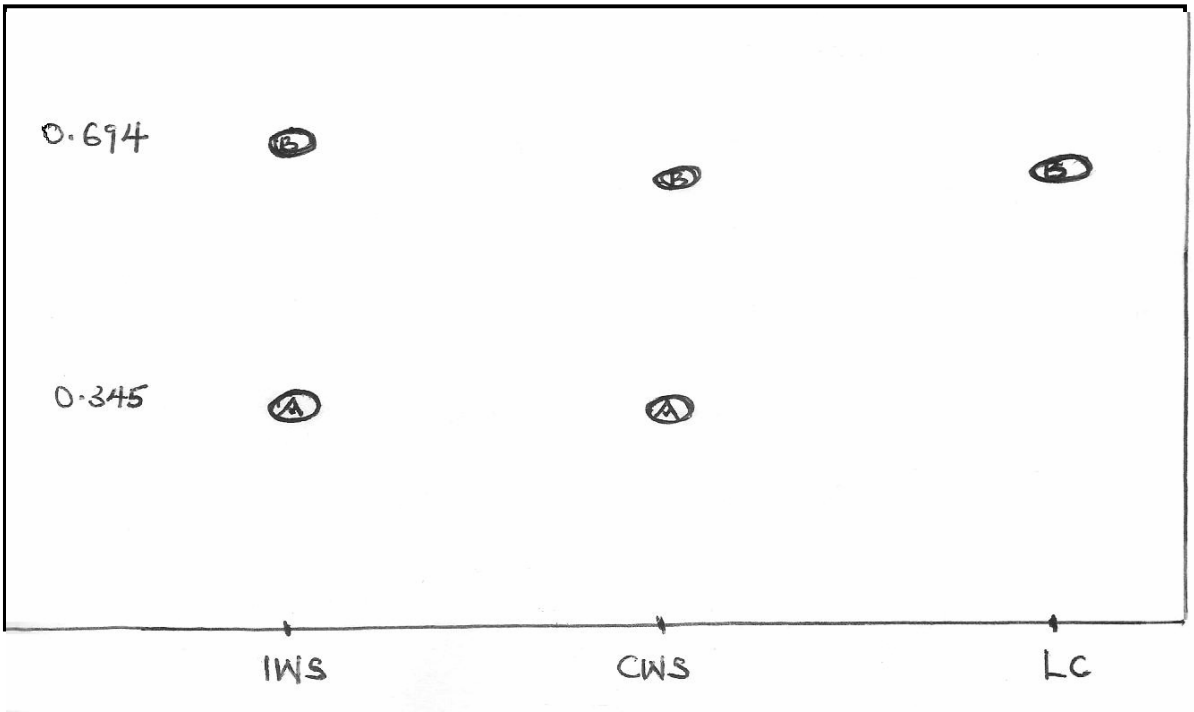


Fig 5: Thin layer chromatography of the metabolites (R_f values)

Spot	R_f
A	0.345
B	0.694

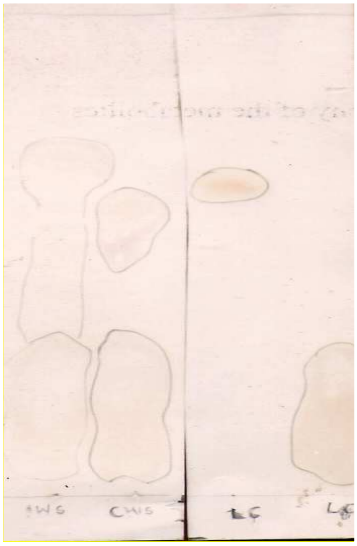
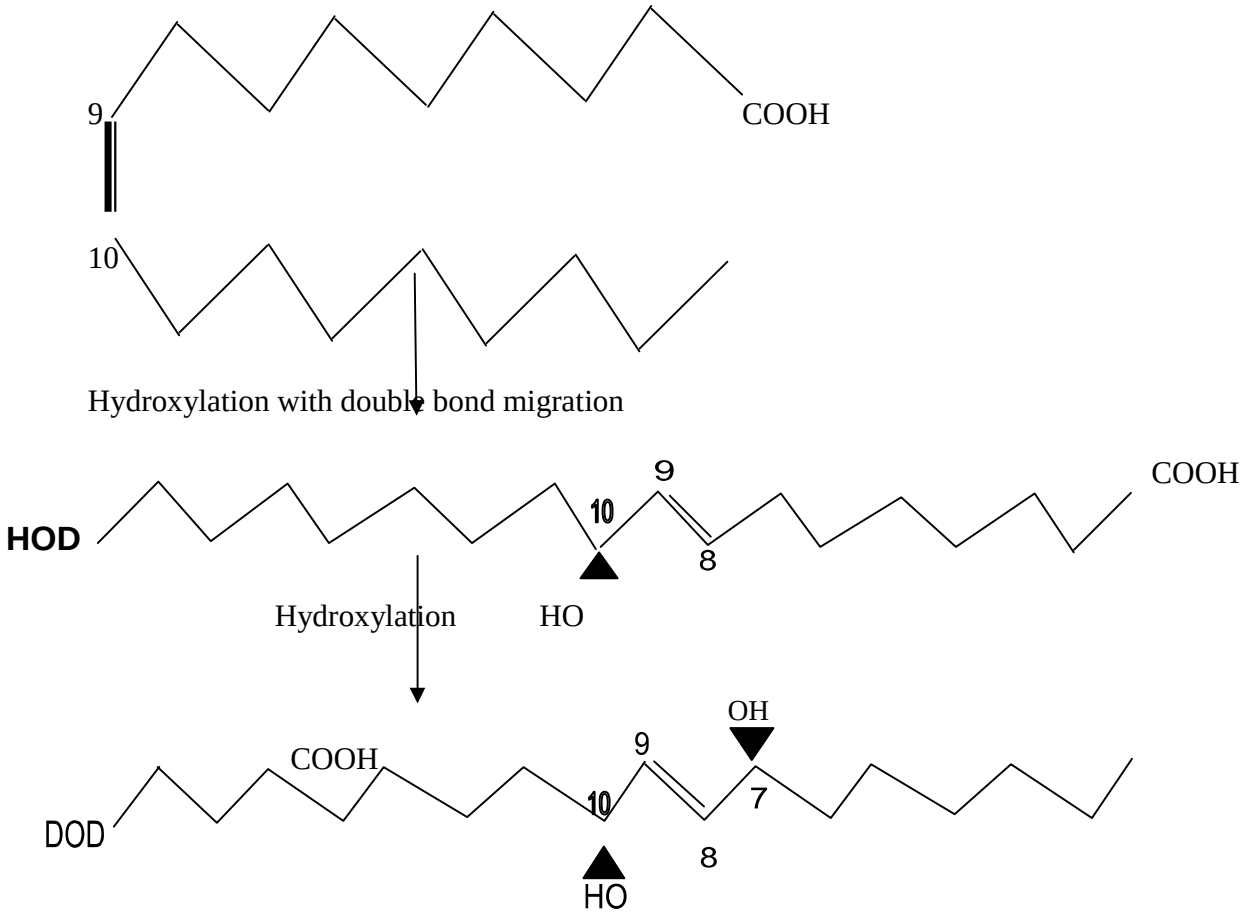


FIG. 6: Thin layer chromatography of the metabolites (spot locations) of IWS,CWS and LC using petroleumether/diethyl ether/glacial acetic acid (90:10:1 by vol) as solvent.

Discussion

Metabolites were prepared from seven organisms. However, the metabolites from *Klebsiella aerogenes* and *Bacillus pumilus* exhibited more antimicrobial activities.The more active metabolites produced by *Klebsiella aerogenes* and *Bacillus pumilus* compared to others are probably because they contain those constituents which enhance antibacterial potency as revealed by the IR analysis, substituents like carboxylic acids, ethyl carbonyl, aldehyde and even aromatic rings that have been known to contribute to antimicrobial properties of metabolites (Figures 3 and 4). The result obtained in the chromatographic analysis of the metabolites of *Klebsiella aerogenes* and *Bacillus pumilus* and the compound with R_F value of 0.345 formed (Figs.5), is closely related to what was obtained in literature by Koritale *et al*., (1989). This compound is suspected strongly to be 10- hydroxyl octadecaenoic acid (spot A) – A novel lipoidal surfactant (Manresa *et al.*,1990).

Hou *et al*., (1993) postulated the pathway for the bioconversion of oleic acid to 10-hydroxyloctadecanoic acid. Oleic acid is first converted to HOD (an intermediate products). During this step, one hydroxyl group is introduced at C-10 (s) and a doubled bond is shifted from C-9 cis to C-8 trans, suggesting that there may be at least 2 or more enzymes involved in this first step for cis-trans- shifted isomerization of the double bond and further hydroxylation introducing a hydroxyl group at C-7 (s). The DOD pathways mentioned below is the bioconversional products for the metabolites produced.



HOD, 10 – hydroxy – 8 – octadecanoic acid

DOD, 7, 10 – dihydroxy – 8 (E) – octadecanoic acid.

Figure 7: A postulated pathway for bioconversion of oleic acid to DOD by strain PR3 from *Pseudomonas aeruginosa* (Hou *et al*., 1993).

The infrared (IR) spectral analyses of the metabolites from the two organisms showed that both have close ranges of absorption. The intense absorption bond at 1745.7 cm^{-1} (Figs.3 and 4) show the presence of ester carbonyl in both samples. Absorptions at 2858.3-2931.3 and 3363 cm^{-1} will produce aldehyde and carboxylic acid respectively which further confirms the presence of carbonyl groups which could either be hydroxyl or ketostearic acids . Based on the spectral measurements and their comparisons with values in literature, the suspicion of 10-hydroxyl octadecaenoic acid in the metabolite is reasonable (Tulloch, 1980; Tulloch, 1985). Antibacterial agents are natural products of bacteria or derivatives of their products which kill (bactericidal) or inhibit the growth (bacteriostatic) of the organisms. It forms part of the self preservation mechanisms by which the microbes prevent overcrowding with their own or other species (Mims *et al.,* 1993). Antibacterial agents are undoubtedly invaluable in the treatment and prevention of infections. But some factors such as use and abuse of drugs, drug resistance bacterial strains, loss of confidence by users, relapsed and re-infection of diseases and high cost of antibacterial agents led to their limitations (Ghoshal *et al.,* 1996).

It can be concluded from all the tests and experiments carried out that the metabolites produced by *Klebsiella aerogenes* and *Bacillus pumilus* had better antimicrobial activities on the test organisms and therefore can serve as effective antimicrobial agents .

ACKNOWLEDGEMENT: I gratefully acknowledge Prof. J.K. Oloke, Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso, Nigeria for his supervision, encouragement and helpful discussion. I thank Mr. S.O.A. Olatope at the Federal Institute of Industrial Research Oshodi (FIIRO) for the characterization of the strains and Dr. Ayodele Elizabeth Omotayo thru Prof. M.O.Ilori both in the Department of Microbiology at the University of Lagos, Nigeria for the realization of this publication.

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