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Full Length Research Paper

Screening of bioactive compound, antimicrobial activity producing halophilic isolates from the saltpans of Thoothukudi district

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Extreme environments harbor a number of microbes producing novel bioactive compounds. The aim of our study is to isolate and identify bioactive compound producing halophiles. Marine soil sediments were collected from the solar saltpans of Thoothukudi District, Tamil Nadu, India. Based on colony morphology, two species were isolated and identification was done by using morphological and biochemical tests. The extracts of cell-free supernatant of the two halophilic isolates were screened for bioactive compound and tested for antimicrobial activity against human pathogenic bacteria such as *Staphylococcus aureus, Pseudomonas* sp, *Klebsiella* sp, *Vibrio* sp, *Escherichia coli* and fungi *Aspergillus niger* and *Penicillium chrysogenum* by the agar cup diffusion method. The results were then compared to standard antibiotics which showed 80% of similar activity in 50 µL/g concentration. In addition, the arbitrary unit of two isolates was calculated against *S. aureus* which produced enhanced inhibitory results. Hence our finding illustrated that Thoothukudi saltpan might be considered as a resource for novel bioactive compounds.

Key words: Halophilic bacteria, bioactive compound, anti-microbial activity, arbitrary unit, Thoothukudi saltpan.

INTRODUCTION

The Earth's surface consists of 70% water, which is inhabited by 80% of all life forms with greater diversity and consequently, marine environment can be described or characterized as a number of different scales, ranging from ocean-level processes which occur at species and genetic level (Bruckner, 2002; Connor et al., 2002).

Marine soil has been widely explored as the source of microorganisms, possessing a large number of bioactive molecules. Halophiles are a group of microorganisms that live in saline environments and are economically important because it produces several bioactive compounds which are useful for many pharmaceutical industries (Ghosh et al., 2010). Among the halophilic microbial forms, the halophilic bacterial forms are mostly known for its secondary metabolites such as proteins, amino acids, etc. Notwithstanding, there is an enormous

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Figure 1. Study area and sampling area - Thoothukudi District.

difficulty in isolation and culturing marine bacteria. Significant progress has been achieved in this field, and investigations of bioactive compounds produced by the marine species are rapidly increasing (Dennis and Shimmin, 1997; Wagner et al., 2002). In recent years, marine microorganisms have become important in the study of novel microbial products exhibiting antibacterial, antiviral, antitumor as well as anti-coagulant and cardioactive properties. Thousands of marine bacilli are known to contain antibiotic substance and less than 1% has been examined for their pharmaceutical activity. Although it was proved that Halophilic bacteria have a potent activity for the production of antimicrobial compounds and their antimicrobial spectrum against pathogenic microorganisms differ.

Thoothukudi district in India possesses considerable theoretical, biological and conservation importance, but the biodiversity of this area is poorly characterized due to a lack of experts and inaccessibility. The present study has investigated the halophilic bacteria for their bioactive secondary metabolites. Therefore, these bioactive compounds might be consider as therapeutics directly or used as lead structures for drug innovation (Proksch et al., 2002; Kamat and Kekar, 2004). Based on the foregoing evidence the present study has been aimed to isolate and identify halophilic bacteria from the saltpan environment of Thoothukudi district and evaluate their potential for the production of bioactive compounds which serves as a source of anti-microbial agents against pathogenic bacteria and fungi.

MATERIALS AND METHODS

Study area

Thoothukudi district is located on the South-East of Tamil Nadu state. The district covers an area of 4621km^2 bounded by the districts of Virudhunagar and Ramanathapuram on the East and Gulf of Mannar on the South East and by Tirunelveli district on the West and South West. Its geographical co-ordinates are 8°47’0’’ North, 78°8’0’’ East (Figure 1).

Sampling area

In this study, the saline soil sample was collected from solar salt pans and coastal areas of Thoothukudi district, Tamil Nadu, India. The collected soil samples were placed in sterile polythene bags and containers and immediately transported to laboratory for further analysis.

Physico – chemical parameter analysis

Physical parameters such as atmospheric temperature, pH and temperature of the brine were analyzed using the standard methods (Strickland and Parson, 1972). Determinations of chemical parameter like sodium, chloride, sulphate, calcium, magnesium, potassium a analysed in soil using standard methods (Vogel, 1978).

Isolation of halophilic bacteria from the saline soil sample

The soil samples were serially diluted in the range of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} in a series of test tubes. Mineral salt (MM63) medium was prepared with an increased concentration of NaCl (6 g / 100 ml). The pH of the medium was adjusted to 7 to 7.4. The samples were spread plated and are incubated at 37°C for 48 h.

Preparation of pure culture of halophilic bacterium species

Mineral salt (MM63) medium slant was prepared in test tubes and the isolated colony obtained in the Petri plates were taken (leaving out mixed culture colony) and streaked in the medium in a zigzag manner. Then test tubes were screwcapped and incubated at 37°C for 48 h.

Identification of the halophilic isolates

Morphological and biochemical studies

Gram staining: A thin smear of the bacterial colony was prepared on a clean slide. The slide was fixed by passing through the flame. The smear was covered with crystal violet for 30 s, and then rinsed with water and air dried. The smear was covered with iodine for 30 s, rinsed with water, decolorized with 95% ethanol and washed with water then, counterstained for about 30 s with safranin and washed with water. The slide was examined under oil immersion (1,000x). Colonial appearances were examined after incubated for 3 to 7 days.

Biochemical tests: The halophilic isolates were screened for many biochemical tests (Indole, methyl-red, voges–proskauer, citrate utilization, motility catalase, oxidase, D-Galactose, D-Fructose, D-glucose, Sucrose and Lactose) using standard procedures of Bergeys Manual.

Solvent extraction method

Two bacterial samples were cultured in nutrient broth containing 6 g/ml of NaCl. The cultures were then centrifuged separately and the supernatant was collected. The cell free supernatants were mixed in three different solvents; Dimethyl sulfoxide (DMSO), ethyl alcohol and phenol separately and the mixture were kept at 4°C overnight. The organic and aqueous phases were tested for antimicrobial activity through well diffusion method (Birbir et al., 2004; Birgul et al., 2002; Gesheva and Vasileva-Tonkova, 2012; Sawale et al., 2014).

Screening of antimicrobial activity producing halophilic bacteria

Anti-bacterial assay

The supernatants of each suspension were assessed for antibacterial property against bioassay strains of bacteria viz., *E.coli*, *Staphylococcus aureus*, *Vibrio sp, Pseudomonas sp*, and *Klebsiella sp* (Pathogens were isolated from the wound samples of infected patients and identified by morphological and biochemical test).

To perform this test, bioassay strain was cultivated on Mueller Hinton agar and wells were made in plate agar using sterile cork borer. 50 and 100 µl of each supernatant were added to each well and plates were incubated at 35°C for 24 h. The exhibition of a clear zone of growth inhibition was observed, measured and quantified which were considered as antimicrobial activity.

Quantification of antibacterial activity

For accurate quantification of inhibition area, the anti bacterial activity score was calculated. Zones of inhibition against various test organisms were measured from the above antibacterial activity in mms and data was computed using the reported quantification procedure (Velho- Pereira and Kamat, 2011) to obtain:

(i) Percent area specific differential antibiotic activity score

$$(PASDASS) = [AWG/TSA] \times 100$$

Where AWG is area on the plate without growth of test pathogen [area of the zone of inhibition-area of the plug (28.26 mm ^{2})] and TSA is the total swabbed area of the pathogen on the plate (Circumference of a Petri plate=6358.5 mm ^{2}).

(ii) Percent overall inhibition efficiency score (POIES), was calculated using the following equation:

$$POIES = (TNIS/TNTS) \times 100$$

Where, TNIS is total number of inhibited species and TNTS is total number of test species. The ideal score for multispecific inhibition would be 100.

Anti-fungal assay

Different fungal cultures (*Aspergillus niger* and *Penicillium chrysogenum*) were swabbed on sterile petri plates containing sterile potato dextrose agar media. The supernatant of cell-free extracts of 50 and 100 µl were then inoculated into the well and tested against the above fungal cultures through well diffusion method. Zone of inhibition around the well was observed, measured and recorded.

Antimicrobial susceptibility assay

The susceptibility of antibiotics test was carried out by the following standard procedure against test organisms onto Mueller Hinton agar plates using antibiotics Streptomycin, Erythromycin and Ampicillin (Dubey and Maheshwari, 2002). The zones of inhibition by the antibiotics were recorded and compared with the antimicrobial activity of the two bacterial isolates.

Arbitrary unit (au) of bioactive compound

To determine an arbitrary unit of the bioactive compound produced by halophilic bacteria isolates, the bacterial culture was serially diluted (10^{-2}, 10^{-4}, 10^{-8}, 10^{-16}, 10^{-32}, 10^{-36}, 10^{-40} and 10^{-52}) then, 100 µl of each dilution was added into wells of seeded Muller Hinton agar by *S. aureus*.

The plates were incubated at 35°C for 24 h and the arbitrary unit of each bioactive compound was determined by the reciprocal of the highest dilution, exhibiting the antimicrobial effect (Hashemi et al., 2014).

RESULTS

Saline soil samples were collected from the coastal areas of Thoothukudi district. Analysis of physicochemical parameters of the marine soil sample is indicated as follows; atmospheric temperature 34°C, temperature of the brine 40°C, pH 7.5, moisture content 30%, ash content 6.4%, Chloride 12.3%, Sulfate 1.63%, Calcium 0.08%, Magnesium 0.54%, Sodium 1.27% and Potassium 0.061%.

Thirty four colonies with two different morphology in mineral salt medium (MM63) were chosen for bacterial isolation. The selected white colored colony was named as GD3007 and the Reddish brown colony was named as DM0207. Different sizes and shapes of colonies were observed after incubation period of 96 h (Table 1). The

Table 1. Colonies and Cell morphology of the halophilic isolates.

S/N	Colony appearance	Isolates named	Cell shape	Gram staining
1	White, mucoid, opaque and translucent	GD3007	Rod	Gram-positive
2	Reddish brown, mucoid, wrinkled with elevated ridges.	DM0207	Rod	Gram-negative

Table 2. Biochemical tests of the halophilic isolates.

Biochemical tests	GD3007	DM0207
Indole production test	-	+
Methyl- Red test	+	+
Voges – Proskauer test	-	-
Citrate utilization test	-	+
Motility test	+	+
Catalase test	+	+
Oxidase test	+	+
D-Galactose	-	+
D-Fructose	+	+
D-glucose	+	+
Sucrose	+	+
Lactose	-	+
Anaerobic growth in DMSO	+	+

isolated pure cultures were subjected to various biochemical characterization and the results were indicated in Table 2. The cell free supernatant extracts were obtained by the above mentioned solvent extraction method which is used in determining anti-microbial activity. The phenolic extract of halophilic isolate GD3007 showed potent activity against all test organisms whereas the halophilic isolate DM0207 inhibited the growth of all test organisms except *S. aureus*. The overall inhibition efficiency score for halophilic isolates (GD3007 and DM0207) was calculated against the test organisms in both 50 and 100 µl (Table 3). Among the three solvent extracts, phenol showed potent activity against the test organisms *A. niger* and *P. chrysogenum* when compared with other two solvents DMSO and Ethyl alcohol in 100 µl (Table 4, Figure 2). The phenol solvent extract showed equal activity as bacterial antibiotics streptomycin, erythromycin and ampicillin and fungal antibiotic ketoconazole in 50 µl. Arbitrary unit of the halophilic isolates (GD3007 and DM0207) was determined against *S. aureus* and the inhibition effect of the test organism was found till dilution factor 10^{-48} for isolate GD3007 and 10^{-52} for isolate DM0207. Phenol solvent extract shows better zone of inhibition activity which indicates that the antimicrobial component may have maximum partition coefficient in phenol.

DISCUSSION

The use of halophilic microorganisms in the industrial application has been increased. It produces a wide range of bioactive compounds such as enzymes (protease, amylase, cellulase etc) (Ganesan et al., 2010), extracellular polysaccharides (EPS), proteins, etc. Marine bacteria were screened for antagonistic activity against terrestrial microbes including *Salmonella typhi, S. aureus, Escherichia coli, Enterobacter aerogenes* and *Streptococcus mutants* (Agricultural Culture Collection of TamilNadu) as test microorganisms.

Antimicrobial activity was determined by using the purified extract which was eluted with ethyl acetate by agar diffusion method using 3 h broth culture which was then compared with MacFarland standard 0.5.

The maximum antibacterial activity was noted in *Bacillus subtilis* (21.5 mm) against *E. coli, B. subtilis* (19 mm) against *S. aureus, Bacillus licheniformis* (18.5 mm) against *S. aureus*, *Bacillus cereus* (16.5 mm) against *E. aerogenes*, *B. licheniformis* (16.5 mm) against *E. aerogenes* .The minimum antibacterial activity were observed in *B. cereus* (14.5 mm) against *S. typhi, Bacillus pumilus* (13.75 mm) against *S. typhi* and *B. pumilus* (13 mm) against *E. coli* (Kannahi and Eshwari, 2016). Same appreciable result was recorded in the present study against different test pathogens.

Antimicrobial activity was assayed in duplicate using a standard paper disc assay (Mearns- Spragg et al., 2012). Anti fungal test was performed by agar well diffusion method against *A. niger* and *Penicillium notatum* by Hashemi et al. (2014). The zone of inhibition was observed after incubating at 27°C for 36 h. In addition, a study demonstrated the inhibitory effect performed till 10^{-}

Table 3. Quantification of antibacterial activity of halophilic isolates (GD3007 and DM0207) in DMSO, ethyl acetate and phenol solvent extract.

Solvent extract	Test organism	Zone of Inhibition in mm (GD3007)				Zone of Inhibition in mm (DM0207)			
		(PASDASS)= [AWG/TSA] x 100 (%)		POIES = (TNIS/TNTS)x 100 (%)		(PASDASS) = [AWG/TSA] x 100		POIES = (TNIS/TNTS) x 100	
		50 µl	100 µl	50 µl	100 µl	50 µl	100 µl	50 µl	100 µl
DMSO	*Escherichia coli*	-	11.9	40	80	6.8	7.9		
	Staphylococcus aureus	8.8	10.8			-	-		
	Vibrio sp	-	-			-	-	60	60
	Pseudomonas sp	8.8	10.8			7.9	16.8		
	Klebsiella sp	-	9.8			8.8	9.8		
Ethyl alcohol	*Escherichia coli*	10.8	10.8			7.9	10.8		
	Staphylococcus aureus	7.9	8.8	80	100	-	-	80	80
	Vibrio sp	8.8	12.8			8.8	10.8		
	Pseudomonas sp	-	8.8			8.8	15.8		
	Klebsiella sp	8.8	10.8			10.8	13.8		
Phenol	*Escherichia coli*	30.8	29.8	100	100	29.8	34.8		
	Staphylococcus aureus	27.8	29.8			32.8	35.8	100	100
	Vibrio sp	24.8	28.8			29.8	32.8		
	Pseudomonas sp	25.8	30.8			34.8	39.8		
	Klebsiella sp	26.8	34.8			34.8	36.8		

Table 4. Antifungal activity of halophilic isolates (GD3007 and DM0207) in DMSO solvent extract.

Solvent extract	Test organisms	GD3007	DM0207
		zone of inhibition in mm	Zone of inhibition in mm
		100 µl	100 µL
DMSO	*Aspergillus niger*	-	-
	Penicillium chrysogenum	-	-
Ethyl alcohol	*Aspergillus niger*	-	12
	Penicillium chrysogenum	-	5
Phenol	*Aspergillus niger*	33	30
	Penicillium chrysogenum	33	32

[128] dilution by *B. licheniformis* against *S. aureus.*

Similarly, the results of the study by Nezami et al. (2016) revealed that the halophilic isolates had the ability to produce secondary metabolites which have bioactivity against human pathogen. Thus halophilic strains finds application in

a. GD3007 (*Aspergillus niger* *Penicillum chrysogenum*) b. DM0207 (*Aspergillus niger* *Penicillum chrysogenum*).

Figure 2. Antifungal activity of halophilic Isolate (a) GD3007 and (b) DM0207 in 100 µL Of DMSO, ethyl alcohol and phenol solvents.

developing pharmacological lead compounds against disease causing human pathogens..

Conclusion

In this present study, two isolates GD3007 and DM0207 showed a wide range of inhibition zone in the primary screening against pathogenic bacteria such as *E.coli*, *S. aureus*, *Vibrio*, *Pseudomonas* and *Klebsiella* and fungi such as *A. niger* and *P. chrysogenum*. The crude extracts of antimicrobial compound were tested for antibacterial activity by well diffusion method. More yield of crude extract was produced with phenol solvents. The reason for the increased yield was due to the lack of water and complete miscibility in organic solvents (Phenol) of the supernatant growth. Thus, the results of this investigation revealed that the marine bacteria collected from the sediments of saltpan might be a potent source of novel antibiotics. Upon conducting tests for bioactive compound production, it was found that they produced bioactive compound against certain bacteria and fungi. The bacteria produced bioactive compounds which are extracellular in nature. Further analysis is needed in future to explore the type of bioactive compounds by isolated microbes and the knowledge which can lead to the discovery of various products that may be of medicinal as well as industrial use.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of indigenous and effective microorganism fertilizers on soil microorganisms and yield of Irish potato in Bambili, Cameroon

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Irish potato (*Solanum tuberosum*) is one of the world's most consumed staple worldwide and an important crop in terms of food security in the face of population growth and increased hunger rates. Potato yields in Cameroon have often been low as a result of decrease in soil fertility. Soil fertility has often been regarded as the chemical and physical properties of soil, with the microbial aspect often being ignored. An experiment was carried out in Bambili, Cameroon to evaluate the effect of two organic fertilizers (indigenous microorganism fertilizer, IMO, and effective microorganism fertilizers, EM) on the yield of Irish potato and to identify some soil bacteria and fungi. A randomized complete block design with three treatments (EM, IMO and control), and four replications was used. Fertilizers were applied one week before planting and repeated four and eight weeks after planting. Soil samples were collected before the application of fertilizers, and then 1, 6 and 10 weeks after application of the fertilizers and used to find out microorganisms present in the different treatments at different periods of plant growth. Different culture media were used for the primary cell culture of the bacteria and fungi using the spread plate technique while isolation of pure bacteria cultures was done by streaking. The fresh weight of tubers under IMO fertilizer was higher than those with EM fertilizer and the control. Some microorganisms identified in the different treatments included: *Aspergillus*, *Rhizopus, Penicillium*, *Fusarium, Saccharomyces, Enterobacteria* and *Pseudomonas* which were present in all the treatments but at different growth stages of the plants. Both IMO and EM fertilizers had significant positive effects on the tuber yield and the soil microbial population in the different treatments.

Key words: Bacteria, fungi, *Solanum tuberosum*, tuber yield.

INTRODUCTION

Microorganisms play an important role in the improvement of soil quality, thereby favoring the growth of plants. In most soil fertility studies, attention is usually focused on soil physical and chemical properties while

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Indigenous microorganisms (IMO) are ‘‘naturally’’ occurring microbes that have adapted to the environmental conditions where they are found and are therefore capable of accelerating decomposition of organic materials found in the same area (Singh and Sharma, 2003).

Effective microorganisms (EM) consist of mixed cultures of beneficial and naturally occurring microorganisms which are applied to the soil in other to increase the microbial diversity of soils and the growth of plants (Muthaura et al., 2010). The concept of EM was first discovered by Higa (Suthamathy and Seran, 2013). EM is used by the crops as a means of improving the efficient utilization of organic matter.

Microorganisms are important attributes in agriculture because they promote the decomposition, cycling and circulation of plant nutrients and reduce the need for chemical fertilizers. Biofertilizers are organic products containing living cells of different types of microorganisms that have emerged as an important component of the integrated nutrient supply system and hold a great promise to improve crop yields through environmentally sustained nutrient supplies (Muthaura et al., 2010). The increased use of chemical fertilizers and some organic fertilizers in agriculture helps the country in achieving self-sufficient food grain production (Sumathi et al., 2012). The soil fertility of an area or location is very important and optimum productivity may turn to long-term economic benefits, which will reflect on the yield and yield components based on the perceived knowledge of soil fertility (Ibeawuchi et al., 2007). Application of organic matter positively affects the growth and development of plant roots and shoots (Ghosh et al., 2004)

EM and IMO are all products of natural farming and have beneficial effects both on the soil and the crops. Notwithstanding, there are differences between them. In terms of number and types of microorganisms found in them, EM has more microbes than IMO. EM has three main families of over 80 different species (Daly and Stewart, 1999). On the other hand, IMO has mainly Lactobacillus and sometimes *Rhizobium* with a few other species (Hiddink et al., 2005). It is easier for farmers to get adequate results while using EM since the microbes are available from a reliable source. In terms of cost, IMO is cost effective than EM since it is collected from the locality (Carandang, 2003). It is less expensive, but with EM, it must be bought from a reliable source. EM has well combined microbes which produce a symbiotic and mutualistic interaction among the constituent microbes (Daly and Stewart, 1999). These microbes therefore work synergistically thereby producing a very effective ecosystem which can ensure survival of most of the microbes. On the other hand, microbes in IMO do not have a mutualistic and synergetic effect like EM as they are collected by chance (Hiddink et al., 2005). In terms of adaptability, IMOs adapt more to the environment since they grow within the same climatic and environmental conditions. Contrary to this, EM are most probably collected from an area with a different climatic and environmental condition. Considering these differences, it is very evident that comparing the effects of EM and IMO on crop productivity will vary according to these differences and it will be difficult to say with precision which fertilizer will produce better results in the soil types.

There are many opinions on what an ideal agricultural system is. Many will agree that it should produce food on a long-term sustainable basis. Others will insist that it should maintain and improve human health, be economical and beneficial to both producers and consumers, actively preserve and protect the environment, be self-contained and regenerative, and above all produce enough food for an increasing world population (Higa, 1991). It will be therefore better for agriculture to be geared towards less chemically intensive to more biologically based practices so as to improve soil health and agricultural production and be less harmful to humans and the environment than conventional agricultural production methods.

A survey carried out in the national territory of 2000 and 2001 showed that potato yields in Cameroon vary according to the production zone, from 3.3 to 6.7 t ha^{-1} with an overall mean of 6.0 t ha^{-1} (Njualem, 2010). In the Western Highlands of Cameroon, it is estimated that over 200,000 small holder farmers, mostly women, are involved in the production of potato. Their production accounts for more than 80% of the national production, estimated at 142,000 t yr^{-1} cultivated on 45,000 ha. In addition, between 1986 and 2009, these farmers were able to increase potato yields from 2.5 to 5 t ha^{-1} (Fontem et al., 2004). The aim of the study was to investigate the effects of indigenous and effective microorganism based fertilizers on soil microbial activities and their effect on the yield of Irish potato.

MATERIALS AND METHODS

Study area

This research was carried out in the research farms of Higher Teachers Training College (HTTC) of the University of Bamenda at the Bambili campus. Bambili is located in Tubah sub-division, Mezam division of the Northwest region of Cameroon. The town has a total surface area of about 250.69 km^2. It is located between latitudes 5° 60’ 0 ’’ and 6° 05’ 0 ’’ north of the equator and between longitudes 10° 12’ 0’’ and 10° 22’ 0 ’’ east of Greenwich Meridian. It has a humid tropical climate with an average annual rainfall of about 2200 mm. The temperature is about 20.7°C (Focho et al., 2009). Bambili has an undulating topography with altitude varying between about 900 and 2270 m above sea level (Yerima and Van Ranst, 2005). The climate is characterized by two distinct seasons: a long wet season (March to October) with high winds followed by a short dry season (November to March) with high light intensity.

Preparation of IMO fertilizer

IMO fertilizer was prepared according to the method of Park and Du

Figure 1. Procedure for collecting microorganisms, (a) Boxes ready to be buried, (b) A box of cooked rice buried, and (c) Rice covered with white mold 7 days after burying.

Figure 2. Culturing the IMO manure, (A) weighing of molded rice, (B) hand kneading of molded rice/sugar mixture, (C) clean clay pot 2/3 full of the rice/sugar mixture

Ponte (2008) using local materials, as elaborated below:

Collection of microorganism from the environment

Five wooden boxes were ¾ filled with steamed rice. The boxes were covered with white paper towel, rubber bands were then used round the top of the boxes to secure the paper towel in place (Figure 1a). The boxes were partially buried, such that their surfaces were left exposed to the atmosphere, and they were covered with fallen leaves (Figure 1b). After 7 days, the boxes were removed (Figure 1c).

The molded rice was transferred from the wooden boxes into a bowl and weighed (Figure 2a). Equal weight of granulated brown sugar was gradually added to the molded rice and the mixture was hand kneaded until it turned uniform, soft and sticky (Figure 2b). A clean clay pot was filled, 2/3 full with the rice/sugar mixture, and covered with paper towel (Figure 2c). The pot was then stored away from direct sunlight for 7 days to allow the mixture to ferment. After 7 days, the pot was removed and water in a ratio of 1:500 (v/g) was added to the fermented mixture. The mixture was then compressed in a 200 L drum, covered with a lid, and kept away from sunlight for another 7 days. After the 7 days, the IMO was ready for use.

Preparation of EM fertilizer

In order to prepare 100 kg of EM fertilizer, 1 kg of brown sugar, 1 L molasses and 1 L of EM inocula were mixed in a clean container using a wooden spoon until a homogeneous solution was obtained (Figure 3a and b). Twenty liters of chlorine-free water was added which served as a favorable medium for the survival of microorganisms. Fifty kilograms of rice and 50 kg of wheat bran were poured on a clean dry cemented floor and mixed thoroughly using a spade. The liquid mixture was then poured in a hole made in the middle of the dry ingredients and mixed with the hands and spade until the mixture was homogeneous (Figure 3c and d). The mixture was then put in a 300 L plastic tank and compressed very well to maintain anaerobic conditions. The tank was properly closed and left unopened for 7 days. At this point, the EM was ready for use.

Land preparation and planting

A piece of land of dimensions 20 m by 15 m was selected using a measuring tape. The land was cleared using a cutlass and tilled using a hoe. A randomized complete block design (RCBD) with three treatments (EM, IMO and the control) and four replications was used. Holes of about 10 cm deep and 30 cm apart were dug and 38 g of EM fertilizer and 38 g of IMO fertilizer was applied according to the treatments one week before planting. It was repeated at 4 and 8 weeks after planting (WAP) round the plant making sure it does not touch the stem. One potato seed (CIPIRA variety) was planted per hole on the same day one week after the first application of treatments.

Harvesting

Harvesting was done at 12 WAP, when the plants had already withered showing that tubers were matured. Tubers from each

Figure 3. Preparation of EM manure. (a): 1 L of molasses and EM, (b) 1 kg of brown sugar mixture, (c) Liquid mixture poured in the rice, (d) Mixing rice and wheat bran.

treatment were counted and weighed.

Collection and preparation of soil samples for microbial analysis

Before the application of the fertilizer, soil samples were randomly collected at a depth of 0 - 15 cm using a sampling auger and then bulked and labeled. This procedure was repeated for different treatments at 1, 6 and 10 weeks after planting on the spots where fertilizers were applied. All samples were stored in sterilized bottles, ready for microbial analysis.

Preparation of culture media

Primary cell culture of bacteria was done as described by Ahmed et al. (2013) in which ten-fold dilution (10^{-4}) was used for bacterial culture following the spread plate technique. One hundred microliters of diluted sample (from 10^{-4}) was pipetted unto the surface of agar. A sterile spreader was used to spread the sample evenly on the entire agar surface. The plates were labelled and incubated at 37°C in a bacteriological incubator (BINDER, USA) for 72 h.

Nutrient agar medium which is a multipurpose medium for bacteria, Sabouraud Dextrose Agar, a multipurpose medium for fungi, Cled Agar medium also for *Salmonella* and *Pseudomonas*, and Mac Conkey Agar medium which is medium for *E. coli, Enterobacteria* and coliforms, were used.

Primary cell culture of bacteria

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Isolation of pure bacterial cultures

The streak plate technique described by Cheesbrough (2000) was used for the isolation of pure bacterial cultures. A sterile inoculation loop was used to pick out small amounts of bacteria from separate morphologically distinct colonies. This was used to inoculate sterile nutrient agar surfaces by streaking. The plates were inoculated and incubated at 37°C in a bacteriological incubator for 24 to 48 h.

Extraction and culture of fungi

Fungi species found in the treatments were identified using the method of Gautam et al. (2011). Chloramphenicol antibiotic (0.03 mg/L) was added to the media to avoid bacterial contamination.

Identification of fungal isolates

Fungal isolates were identified as described by Navi et al. (1999) on the bases of morphological and microscopic examinations.

Morphological characterization of bacterial isolates

Isolated bacteria were characterized based on colony and cellular morphology as described by Cheesbrough (2000).

Characterization by colonial morphology

Colonial morphology was described using parameters such as colony form, margin, colour, elevation and appearance. Freshly cultured bacterial isolates (24 to 48 h cultures) were characterized morphologically by observing and recording the above colonial parameters.

Characterization by cellular morphology

Characterization by cellular morphology was carried out following Gram's staining. This was done as described by Cheesbrough (2000). Freshly cultured bacteria isolates (48 h cultures) were used for these purposes.

Gram stain

Preparation and fixing of smears

With the use of a sterile inoculation loop, 2 to 3 loopful of sterile distilled water was placed on a clean dry labeled grease-free slide.

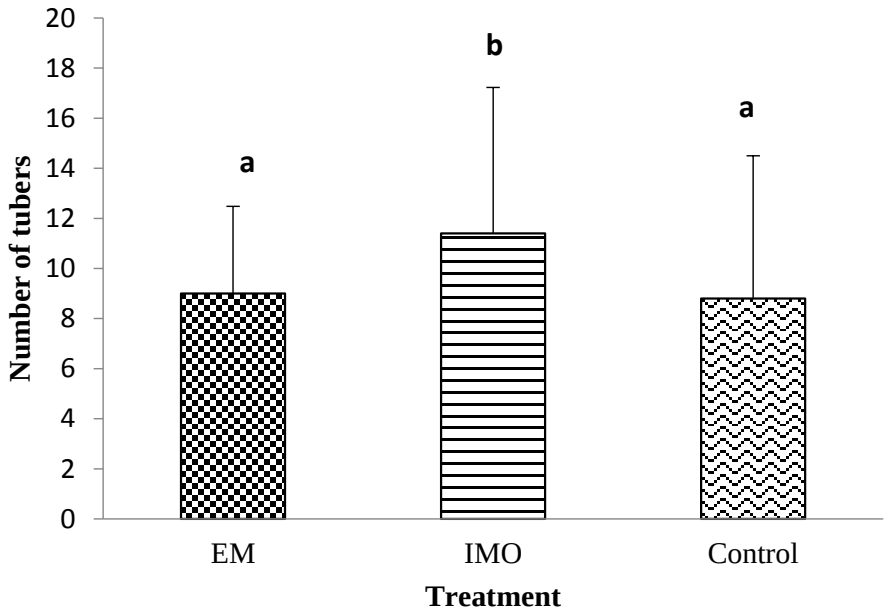

Figure 4. Average number of tubers per plant and per treatment. Histograms with same letter are not significantly different at P ≤ 0.05 (DMRT).

The loop was re-sterilized by heating in a Bunsen flame till it became red hot, and then cooled and used to pick up a small amount of bacteria from single colonies. The bacteria were then emulsified in saline water on the slide to form a thin smear. The slides were air-dried completely. Smears were fixed by rapidly passing the slide, smear uppermost, three times through the flame of a Bunsen burner. The smear was allowed to cool before staining.

Gram staining procedure

The fixed smear was flooded with crystal violet solution for 30 to 60 s. The dye was quickly drained and washed with clean running water. The smear was then covered with Lugol's iodine for 30 to 60 s. The iodine was drained and slide washed gently using clean running water. Rapid decolourization of the smear was done using acetone-alcohol for 5 s and slide washed gently using clean running water. The counterstain, carbolfuchsin was used to flood the slide for 30 s after which it was drained and washed with clean running water. The slides were then dried in a preheated oven for 5 min and then observed under oil immersion lens (100x objective). Bacteria cells were then characterized as either Gram-positive if they stained dark purple or Gram-negative if they stained pink.

Identification of bacteria strains

The different bacteria strains were identified using the identification technique of Cheesbroug (2000).

Statistical analysis

The data collected were analyzed using Microsoft Excel (2010 version). Data obtained were expressed as means ± SD and analyzed statistically using SPSS statistical software version 17.0 (SPSS Inc., Chicago). Significant differences between mean values were determined using analysis of variance (ANOVA). Duncan multiple range Test (DMRT) was used to compare treatment means at 0.05 level of significance.

RESULTS

Number of tubers per plant

Plants treated with IMO fertilizers produced the highest number of tubers per plant (11.40 ± 5.83), followed by those treated with EM manure (9.00 ± 3.48), and then the control plants, the least (8.80 ± 5.69). The number of tubers of plants treated with IMO was significantly different from the number of tubers of plants treated with EM manure and the number of tubers of control plants (Figure 4).

Fresh weight of tubers

Plants treated with IMO fertilizers produced potato tubers with the heaviest weight (241.64 ± 32.94 g), followed by those treated with EM manure (227.62 ± 44.58 g) and the control which produced tubers with the least weight (125.66 ± 31.63 g). Statistical analysis revealed significant differences (p ≤ 0.05) between plants treated and control plants (Figure 5).

Morphological identification of fungi

The fungi were colonially and microscopically identified and found to belong to the same phylum of Ascomycota,

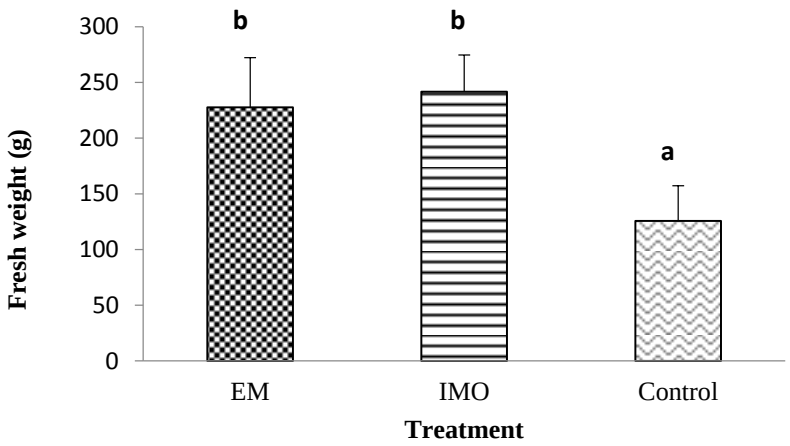

Figure 5. Average weight of tubers per treatment. Histograms with same letter are not significantly different at P ≤ 0.05 (DMRT).

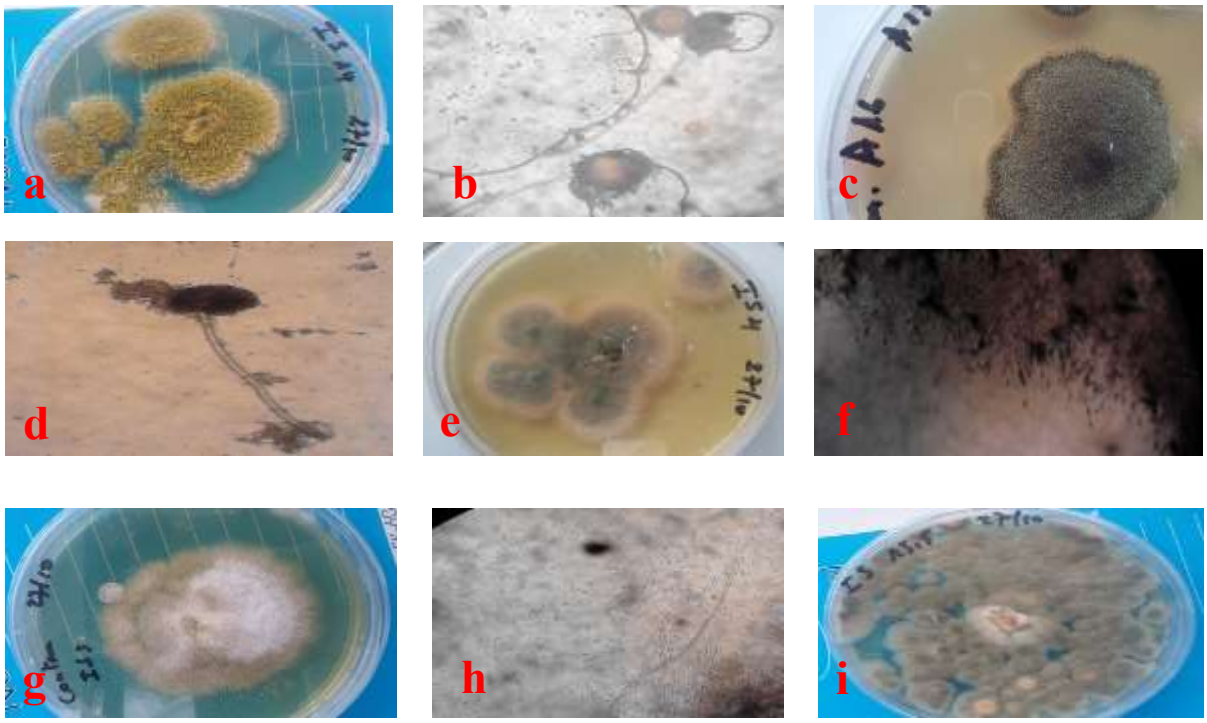

Figure 6. Surface and microscopic view of fungi in different treatments; (a, b) *Aspergillus*, (c, d): *Rhizopus*, (e, f): *Penicillium*, (g, h): *Fusarium*, (i):*Saccharomyces.*

but from five genera (*Aspergillus*, *Rhizopus, Penicillium*, *Fusarium* and *Saccharomyces*, respectively) (Figure 6).

Characterization of bacterial isolates by colonial morphology

A total of 6 distinct bacterial isolates were obtained in the soil samples. These isolates were distinguished based on their colonial morphology observed on nutrient agar plates (Table 1).

Characterization of bacterial isolates by cellular morphology

Of the six isolates, two (I_5 and I_6) were Gram positive rods with I_6 having central spores. The remaining

Table 1. Morphological characterization of bacteria isolates.

Parameter	I_1	I_2	I_3	I_4	I_5	I_6
Margin	Entire	Undulate	Undulate	Entire	Entire	Undulate
Form	Circular	Irregular	Irregular	Circular	Circular	Irregular
Elevation	Raised	Raised	Flat	Convex	Flat	Undulate
Colour	Pale orange	Dark green	Creamy	Creamy	Brick red	Creamy
Appearance	Mucoid	Mucoid	Dull	Mucoid	Mucoid	Dull

I = isolates.

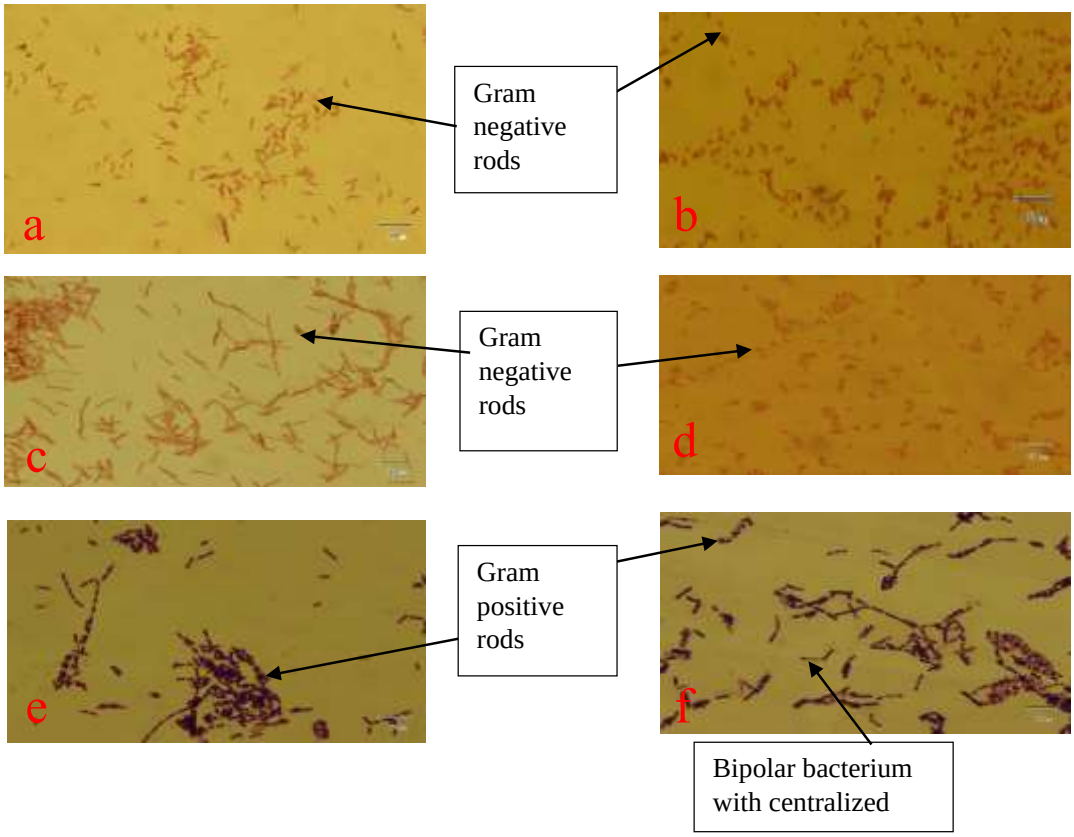

Figure 7. Gram stains of bacteria isolates in different treatments; (a) Gram stain of I1, (b) Gram stain of I2, (c) Gram stain of I3, (d) Gram stain of I4, (e) Gram stain of I5, (f) Gram stain of I6.

fourwere Gram negative rods (Figure 7).

Fungi and bacteria found in the different soil treatments with time

Aspergillus was found in both IMO and EM soil at 6 WAP but at 10 WAP, it was only present in the IMO soil. Before the application of the different treatments, it was not present and throughout the growth period of the plants, it was absent in the control. *Rhizopus, Penicillium and Fusarium* were absent before the application of treatments and *Rhizopus* was present only at 6 WAP for the control, while for IMO, it was present throughout and absent at the 10th WAP for EM. *Saccharomyces* were present in all the different treatments before and after application of the manures (Table 2).

From the Gram staining results, the bacteria were identified to belong to two main groups: the *Enterobacteriaceae* which were the Gram negative rods and the *Pseudomonas* which were Gram positive rods. *Enterobacterium* was present in all the different treatment before and after application of the manures. *Pseudomonas* was not in the soil before the application of fertilizers and at 1 WAP. It was present in the control

Table 2. Variation in some fungi and bacteria found in the different soil treatments with time.

Microorganism	Before	1 WAP			6 WAP			10 WAP		
		E	I	C	E	I	C	E	I	C
Aspergillus	-	-	-	-	+	+	-	-	+	-
Rhizopus	-	+	+	-	+	+	+	-	+	-
Penicillium	-	-	-	+	-	-	-	-	-	-
oFusarium	-	-	-	+	+	+	+	+	+	+
Saccharomyces	+	+	+	+	+	+	+	+	+	+
Enterobacteriaceae	+	+	+	+	+	+	+	+	+	+
Pseudomonas	-	-	-	-	+	+	-	+	+	+

E = EM, I = IMO, C = control, + = present, - = absent.

only at 10 WAP, while for EM and IMO soil, it was present at 6 and 10 WAP (Table 2).

DISCUSSION

The crop yield was greater in the treated soil than the control soil with IMO manure having a greater and more significant tuber yield than EM fertilizer. The number of tubers per plant and fresh weight of tubers were higher in IMO and EM treated soils. EM and IMO manures are organic fertilizers which play a significant role in maintaining and improving the chemical, physical and biological properties of soils and in sustaining crop yield. Beneficial microorganisms in IMO were indigenous to the soil and environmental conditions of the farm and could more easily adapt, unlike those in EM manure which were only imported from abroad (Prell, 2010). According to Koon-Hui et al. (2013), IMO treated plants did best because mycorrhizae contributed to the soil tilt in IMO plots. This result is confirmed by Woo et al. (2006) with the explanation that beneficial fungal species colonize plant root and stimulate increased nutrient uptake to improve yield (Muyang et al., 2014). However, this result was different from the observations made by Yamada and Xu (2000) where EM treated plants did better than IMO treated plants. Mbouobda et al. (2014) showed that carrots grown with EM manure did better than those grown with IMO manure and the control.

Aspergillus, Penicillium, Enterobacteria and the *Pseudomonaceae* are amongst the most powerful phosphate solubilizers (Whitelaw et al., 2000). *Aspergillus* was found in both IMO and EM soil at 6 WAP and at 10 WAP, it was only in the IMO soil. Before the application of the different treatments, it was not present and throughout the growth period of the plants, it was absent in the control. This was similar to studies carried out by Gaur and Sadasivam (1993) where the nutrient status of sorghum stalk and wheat straw compost was improved when inoculated with *Aspergillus niger* and *Penicillium* species. However, it was not the case with *Penicillium* which was present only in the control at 1 WAP. *Rhizopus* is a type of mold which grows on organic matter and could be the reason why it appeared more in the inoculated soils. *Fusarium* was present only in the control at 1 WAP and in all the treatments at both 6 and 10 WAP. *Fusarium* is a plant pathogenic microorganism which causes wilting in potatoes. When a soil has a high population of *Fusarium, Phytophthora* and *Pythium*, it is considered to be a disease inducing soil (Higa, 1994). *Saccharomyces* was present in all the different treatments before and after application of the fertilizer. When microbial amendments are applied to the soil, their fermentative activities can increase drastically and overwhelm the indigenous soil microflora for an indefinite period (Higa and Parr, 1994). *Enterobacterium* was present in all the different treatments before and after application of the manures. *Pseudomonas* was not present before the application of the fertilizer and at 1 WAP. It was present in the control only at 10 WAP while for EM and IMO soil, it was present at 6 and 10 WAP. *Enterobacteria* and *Pseudomonas* are phosphate solubilizers which play an important role in supplementing phosphorus to plants (Tamberkar et al., 2009). This also explains why the yield was higher in the two amended soils as compared to the control.

Conclusion

The purpose of this study was to evaluate the effect of IMO and EM fertilizers on the yield of Irish potato (*Solanum tuberosum*) and on some of the microorganisms in the soil. From this study, the following conclusions were drawn:

(i) Both IMO and EM fertilizers had a better effect on the yield of Irish potato with IMO producing the best yields in terms of number and weight of tubers.
(ii) *Aspergillus, Penicillium, Enterobacteria* and *Pseudomonaceae* were the most common microorganisms and are amongst those that help in improving crop productivity.

According to the results obtained, the use of

microorganisms (indigenous and effective microorganism manure) was shown to significantly increase the yield of Irish potato and therefore could be recommended as organic amendment for improving yield of Irish potato.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Ameliorative effect of imipenem on pulmonary damage caused by extended spectrum β-lactamase (ESBL) producing bacteria isolates from non- human source

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Acute lung infection induced by Extended Spectrum β-Lactamases (ESBL) producing isolates was determined by measuring inflammatory mediators; malondialdehyde (MDA), myeloperoxidase (MPO) and nitric oxide (NO). The mice were randomly divided into three groups of 20 animals each. All mice were given 10^4 c.f.u. ml^{-1} of the test organism intranasally in a volume of 50 µl while holding the mouse in an upright position without any anaesthesia. Group A received an intraperitoneal injection of an antibiotic, imipenem at a dose of 20 mg ml^{-1}/ 25 g body weight which was administered 48 h post infection, Group B received only normal saline orally while group C, control-mice did not receive any treatment. The animals were sacrificed by cervical dislocation; lungs were removed aseptically and examined for various inflammatory mediators. The MDA, NO and MPO estimations in the lung homogenates in each group was measured and compared. Group treated with imipenem recorded lower absorbance values when compared with group treated with normal saline. The different parameters were statistically significant since the P-values were less than 0.05.

Key words: Isolates, malondialdehyde, myeloperoxidase, nitric oxide, inflammation, imipenem.

INTRODUCTION

Animal flesh is considered one of the most nutritive sources of proteins consumed by humans. The meat is rich in proteins with high levels of amino acids and polysaturated fats (Listratet et al., 2016). Its water content also makes it a good substrate for microbial growth. Tissues of most healthy animals are sterile but may get

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contaminated by microorganisms during slaughter, dressing and cutting through the knives and other equipment used, through the exterior of the animal, the intestinal tract, the air and the handlers (Cook et al., 2017). The extrinsic factors that determine microbial growth are temperature, moisture content and oxygen availability (Gundogan et al., 2011). It has been reported that Gram negative bacteria account for approximately 69% of bacterial food borne diseases (Cook et al., 2017). A number of foods (meat inclusive) have been reported to have high incidence of bacteria (Overdevest et al., 2014; Casella et al., 2015; Belmahdi et al., 2016). There is, however, limited information on the health implications of food borne diseases associated with ESBL producing organisms. Extended Spectrum β-Lactamases (ESBL) are a group of β-Lactamases which have the ability to hydrolyze third generation cephalosporins such as cefotaxime, ceftazidime and monobactams such as azetreonam (Bush, 2008). They are an increasingly important cause of multidrug resistance in Gram-negative bacteria throughout the world and have coevolved with the β-lactam antibiotics ever since they came into clinical use (Bush and Jacoby, 2010; Doi et al., 2013; Adesokan et al., 2015). The spread of ESBL-producing organisms is greater in developing economies due to poor personal and environmental hygiene conditions; extensive self-treatment, use of non-prescription antimicrobials, very low infection control methods and other numerous reservoirs such as; water, soil, animals, pets and food (Chong et al., 2013; Tansarli et al., 2014; Adenipekun et al., 2015). Animal models of infections caused by ESBL producing organisms have served as a guide to test the efficacy of mono and combination therapies *in vitro* but *in vivo* evaluation in some cases give surprising contrast to results obtained *in vitro* (Bali et al., 2016; Overdevest et al., 2014). All the isolates used in this study are associated with the environment; they are ubiquitous in nature and have the ability to survive long stretches of time under highly desiccated conditions on abiotic surfaces. To the authors knowledge, there is no literature on ESBL induced pulmonary damage in Nigeria, hence, this study was aimed at determining oxidative stress and other inflammatory markers induced by ESBL producing organisms and the effect of imipenem- a carbepenem as a treatment option.

MATERIALS AND METHODS

Extended Spectrum Beta Lactamase (ESBL) producing isolates of *Stenotrophomonas maltiphilia, Acinetobacter baumanii, Pseudomonas monteilli, Achromobacter ruhlandii* and *Pseudomonas fulva* obtained from swab samples of the intestine of animals killed on the spot in the abattoir and from the surfaces of tables where the meat is sold in an abattoir were used for this study.

Induction of acute lung infection

Healthy albino mice of either sex, 6 to 8 weeks old, weighing 20 to 25 g were used. Acute lung infection in mice was induced with test organism, following the modified method by Yadav et al. (2003). A single isolated colony of test organism obtained on a nutrient agar plate was transferred to 50 ml nutrient broth and incubated at 37°C for 18 h. Bacterial cells were harvested by centrifugation at 5000 rpm. for 15 min. The bacterial pellet so obtained was given three washings with sterile PBS (0.1 M, pH 7.2).

The final pellet was suspended in a minimum volume of Phosphate Buffer Solution (PBS) (0.1 M, pH 7.2) to get the desired concentration (OD_{600} = 0.03 Ω 10^4 c.f.u. ml^{-1}). The mice were randomly divided into three groups (A - C) and each group comprised 20 animals. Groups A, B and C were given 10^4 c.f.u. ml^{-1} of the test organism intranasally in a volume of 50 µl while holding the mouse in an upright position without any anaesthesia. After infection, animals were sacrificed on different days post-infection after drug administration by cervical dislocation and lungs were removed aseptically and examined for various inflammatory parameters.

The three groups were given one of the following treatments:

A. Mice infected received an intraperitoneal injection of an antibiotic, imipenem at a dose of 20 mg ml^{-1}/ 25 g body weight; it was administered 48 h post infection with test organisms.
B. Mice infected with 10^4 cfu/ml of the test organisms in a volume of 50 µl received only normal saline orally and no standard drug treatment.
C. Control- Mice were infected but received no treatment.

Quantitative bacterial count in lungs

Mice were sacrificed on different days post-infection by cervical dislocation; lungs were removed aseptically and then homogenized in 1 ml normal saline. Serial dilutions of the homogenized lung tissue were made and plated on nutrient agar plates to determine bacterial load. The plates were incubated at 37°C for 24 h.

The lung homogenate from each mouse was also processed for the following parameters.

Malondialdehyde (MDA) estimation

The extent of tissue damage in terms of lipid peroxidation was estimated by measuring the amount of MDA by the method of Ohkawa et al. (1979). In brief, 0.2 ml of the lung homogenate was mixed with 4 ml 0.045 M sulphuric acid, 1.5 ml of freshly prepared 0.8% thiobarbituric acid and 0.2 ml 8.1% SDS.

This mixture was then kept in a boiling water bath for 1 h. After cooling the mixture under tap water, 5.0 ml butanol: pyridine (15:1) was added and the mixture was shaken vigorously. The contents were centrifuged at 4000 r.p.m. for 10 min, the upper organic layer was taken in a separate tube and its A_{532} was taken.

Myeloperoxidase (MPO) estimation

Pulmonary neutrophil infiltration was quantified by measuring the MPO activity using a spectrophotometric method as proposed by Greenberger et al. (1995). Briefly, the lungs were removed, weighed to determine the wet weight and then homogenized in 2 ml homogenizing solution containing 50 mM potassium phosphate buffer (pH 6.0) with 0.5% hexadecyltrimethylammonium bromide and 5 mM EDTA. The homogenate was sonicated and centrifuged at 15,000g for 15 min. The supernatant was mixed in a ratio of 1:15 with assay buffer comprising 100 mM potassium phosphate buffer (pH 6.0), 0.167 mg o-dianisidine hydrochloride ml^{-1} and 0.0005% hydrogen peroxide. MPO activity was assayed by measuring the change in A_{460} from 0 min to 4 min over intervals of 30 s.

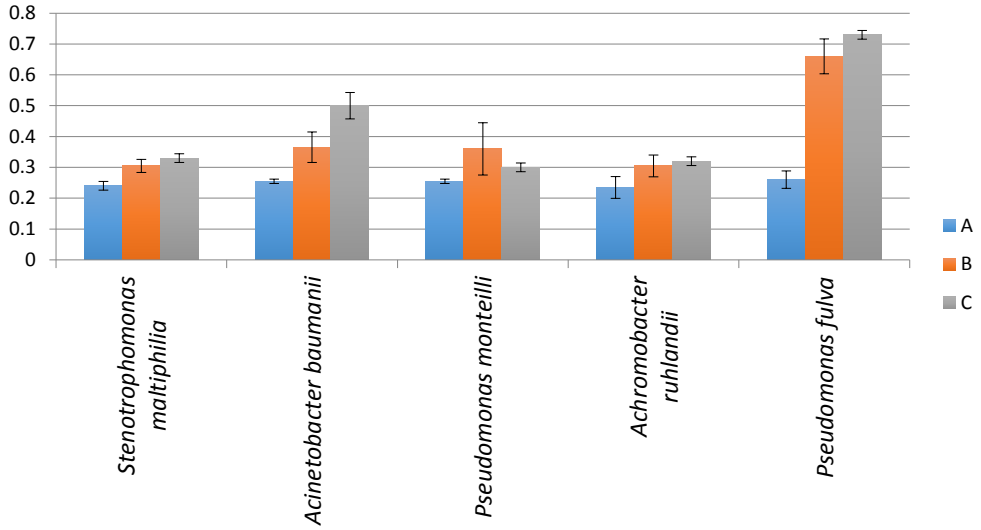

Figure 1. Effect of imipenem and normal saline on malondialdehyde (mda) level in the lung homogenates of mice infected with test organisms measured at (A_{540}). **A** = Absorbance of lung homogenate of infected mice treated with imipenem; **B** = Absorbance of lung homogenate of infected mice treated given normal saline; **C** = Absorbance of lung homogenate of infected mice without treatment.

Nitric oxide (NO) estimation

The nitrite level was estimated in the lung homogenate according to the method of Tsai et al. (1997). Lung homogenate (0.1 ml) was mixed with 0.4 ml PBS (0.1 M, pH 7.2) and 2 ml Griess reagent. Then 2 ml trichloroacetic acid was added and the mixture was vortexed and incubated for 20 min. The mixture was then centrifuged at 14000 g for 10 min and the A_{540} of the supernatant was taken. The nitrite concentration was determined from a standard curve prepared with 100 mM sodium nitrite.

Statistical analysis

Results were analyzed statistically by applying two way ANOVA for comparing various parameters in treated and untreated control rats. Differences were considered statistically significant if P-values were less than 0.05. The statistical software used was SPSS version 20.0.

Null hypothesis

There is no difference in effect between the rats treated with the antibiotic and the rats which received normal saline.

Alternative hypothesis

There is difference in effect between the rats treated with antibiotic and the rats which received normal saline.

RESULTS

Effect of imipenem and normal saline on malondialdehyde (MDA) and nitric oxide (NO) level

Figure 1 shows the different absorbance values for the MDA levels in the lung homogenate of mice infected with test organisms. Mice infected with *Stenotrophomonas maltiphilia* showed a reduction in MDA levels when imipenem was administered. Mice infected with the other test organisms also showed a reduction in lung injury as the MDA levels were also reduced when treated with imipenem except for mice infected with *Pseudomonas monteilli* where treatment with normal saline showed greater efficacy. Lung injury caused by intranasal injection of the mice with the Extended Spectrum Beta Lactamase (ESBL) producing isolates resulted in a significant increase in neutrophil infiltration in the lungs along with increased production of nitric oxide (NO) to cellular injury. Figure 2 shows the treatment response observed. Treatment with imipenem and normal saline had different effects on the mice post infection. The nitric oxide level was highest when the mice were treated with normal saline except in *S. maltiphilia* and nitric oxide levels remained lowest in the control mice. The effect of time on myeloperoxide (MPO) estimation of lung homogenate of mice infected with different test organisms was determined. Figures 3 to 7 shows the myeloperoxide (MPO) level for each of the isolates. Myeloperoxidase is a phagocytic enzyme found in granulocytes that form halogen ions (OCl^-) that are strong oxidizing agents. The effect of time on the myeloperoxidase (MPO) level was determined in the different mice groups for infections caused by the ESBL isolates. There was an increase in MPO level in the mice infected with *S. maltiphilia* due to lung injury. Treatment with normal saline had no significant effect because an increase in MPO level was observed.

Mice group treated with imipenem drastically reduced

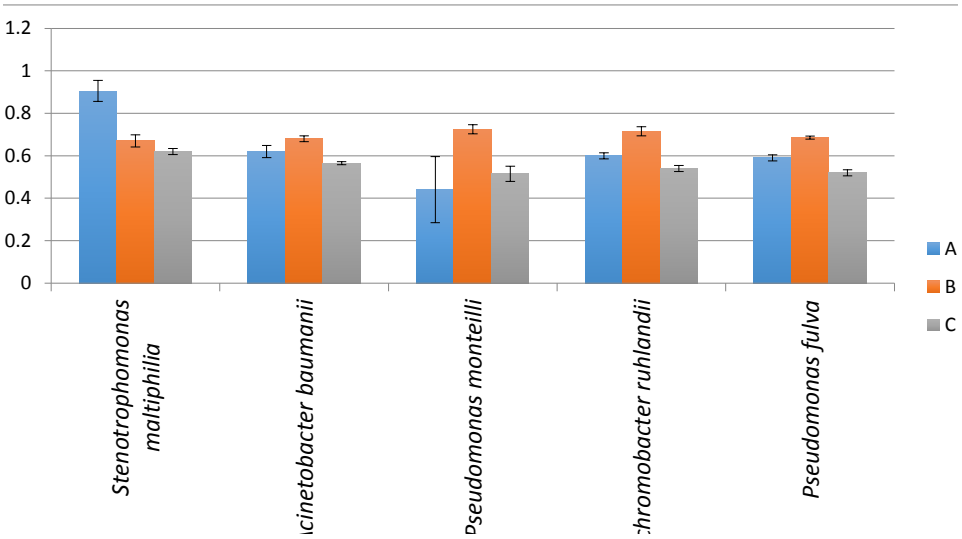

Figure 2. Effect of imipenem and normal saline on nitric oxide (no) level in the lung homogenates of mice infected with test organisms measured at (A_{540}). A = Absorbance of lung homogenate of infected mice treated with imipenem; B = Absorbance of lung homogenate of infected mice treated given normal saline; C= Absorbance of lung homogenate of infected mice without treatment.

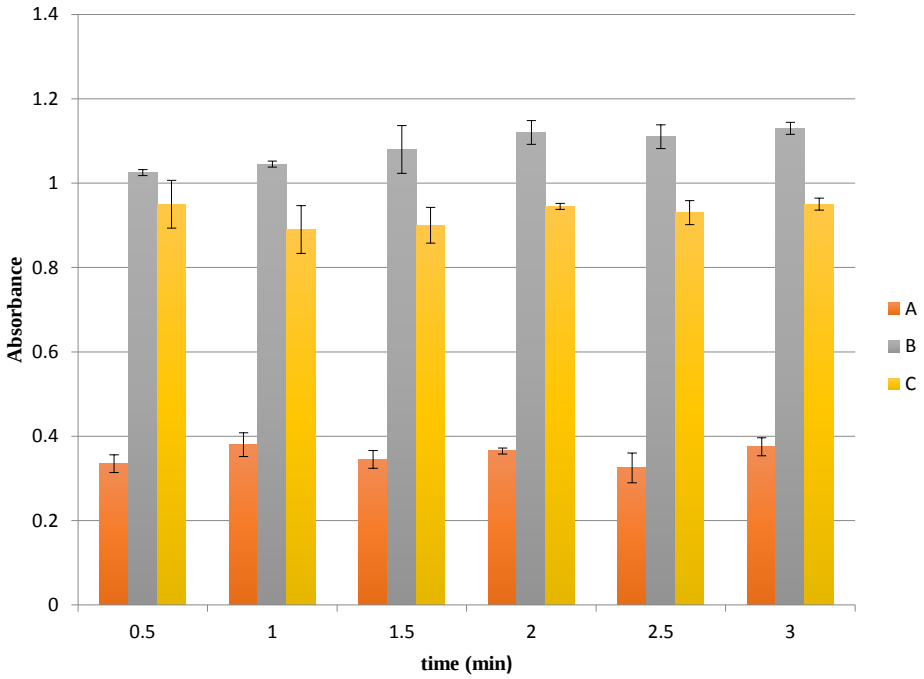

Figure 3. A graph of absorbance against time in myeloperoxide (mpo) estimation of lung homogenate of mice infected with *Stenotrophomons maltiphilia* measured at (A_{460}). **A** = Absorbance of lung homogenate of infected mice treated with imipenem; **B** = Absorbance of lung homogenate of infected mice treated given normal saline; **C**= Absorbance of lung homogenate of infected mice without treatment.

the MPO level which signifies a reduction in the bactetrial load, that is, inhibition of bacterial proliferation which caused the initial MPO influx to the site of injury. Time had no effect on the MPO level since it remained

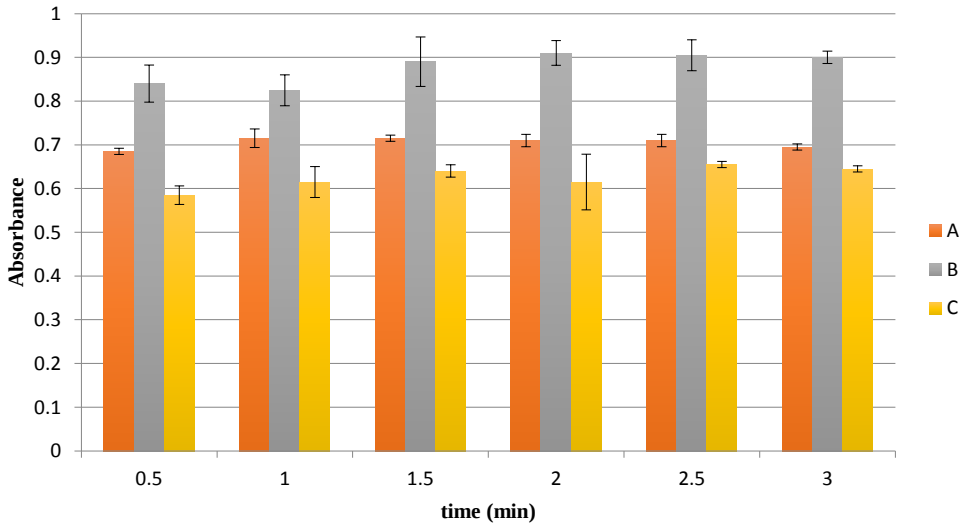

Figure 4. A graph of absorbance against time in myeloperoxide (mpo) estimation of lung homogenate of mice infected with *Acinetobacter baumanii* measured at (A_{460}). A = Absorbance of lung homogenate of infected mice treated with imipenem; B = Absorbance of lung homogenate of infected mice treated given normal saline; C = Control: Absorbance of lung homogenate of infected mice without treatment.

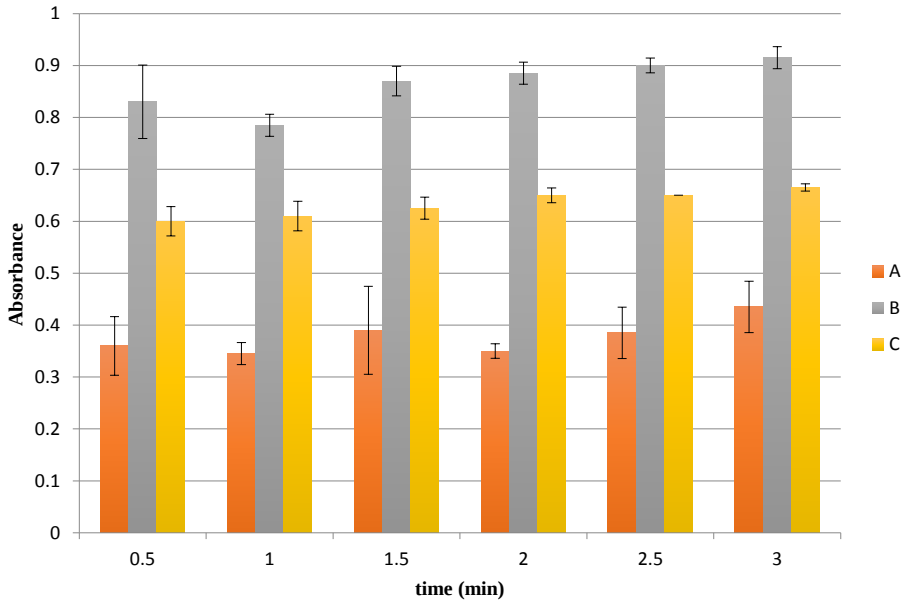

Figure 5. A graph of absorbance against time in myeloperoxide (mpo) estimation of lung homogenate of mice infected with *Pseudomonas montelli* measured at (A_{460}). **A**= Absorbance of lung homogenate of infected mice treated with imipenem; **B** = Absorbance of lung homogenate of infected mice treated given normal saline; **C** = Control: Absorbance of lung homogenate of infected mice without treatment

constant when measured at different time intervals. Infections caused by *Acinetobacter baumanni, Achromobacter ruhlandii* and *Pseudomonas* species increased the MPO levels when normal saline was administered post infection as compared to the control group.

Treatment with imipenem reduced the MPO level when inflammatory property compared with the results of treatment with normal saline but was higher than the control group.

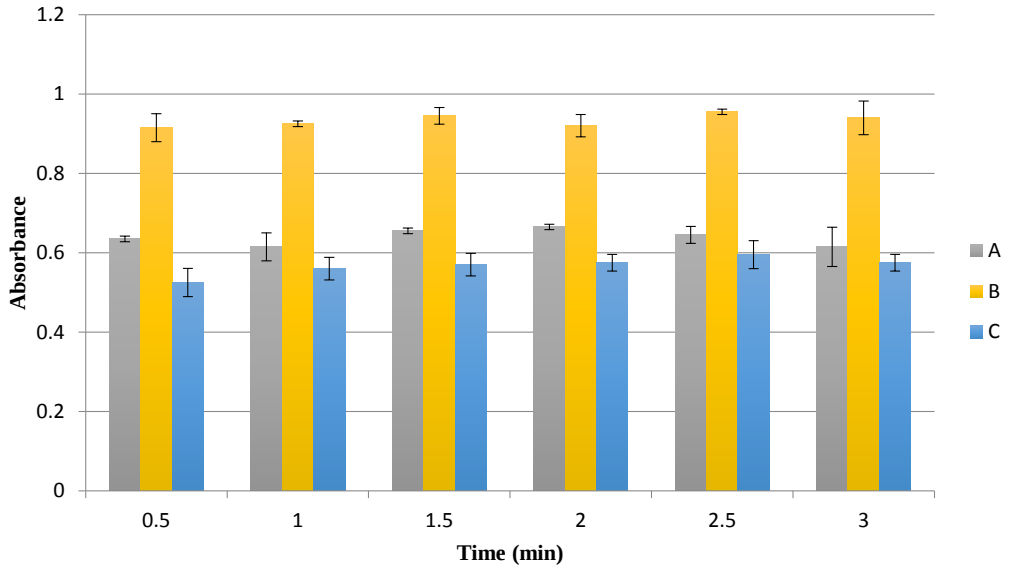

Figure 6. A graph of absorbance against time in myeloperoxide (mpo) estimation of lung homogenate of mice infected with *Achromobacter ruhlandii* measured at (A_{460}). A = Absorbance of lung homogenate of infected mice treated with imipenem; B = Absorbance of lung homogenate of infected mice treated given normal saline; C = Absorbance of lung homogenate of infected mice without treatment.

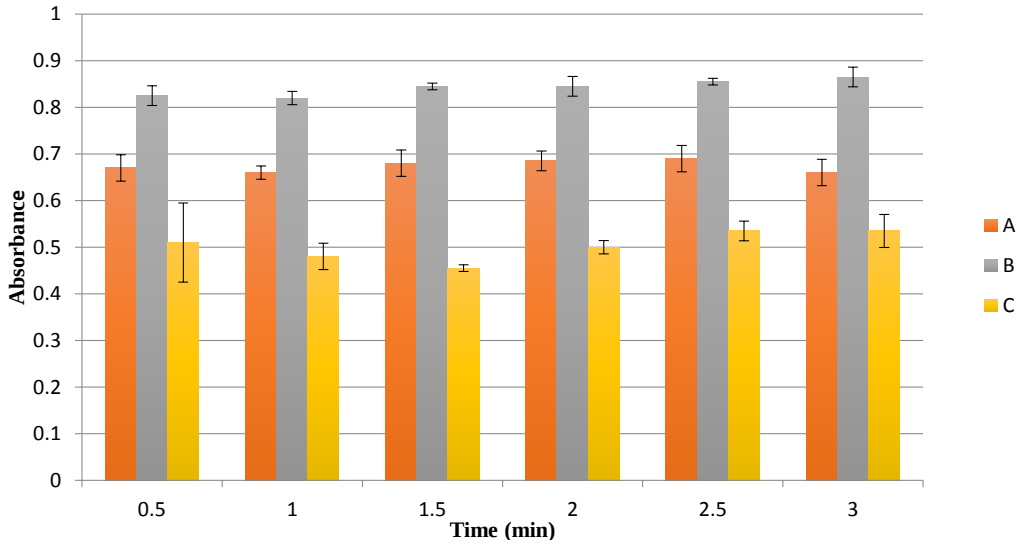

Figure 7. A graph of absorbance against time in myeloperoxide (mpo) estimation of lung homogenate of mice infected with *Pseudomonas fulva* measured at (A_{460}). A = Absorbance of lung homogenate of infected mice treated with imipenem; B = Absorbance of lung homogenate of infected mice treated given normal saline; C = Absorbance of lung homogenate of infected mice without treatment.

DISCUSSION

An attempt was made in this study to confirm the effect of imipenem in an acute lung injury model. On the basis of the results of all the different parameters studied, induction of acute lung inflammation by ESBL producing isolates was observed. Acute inflammation in the lungs is characterized by increased activity of neutrophils (Yamamoto and Pop-Vicas, 2014; Bali et al., 2016). The migration of neutrophils at the site of acute inflammation involves the activity of various inflammatory cytokines and chemokines, and expression of various cell leukocyte

and endothelial adhesion molecules (Bansal and Chhiber, 2010; Mehmet et al., 2013). These facts are further confirmed by the production of the inflammatory parameters namely; malondialdehyde (MDA), myeloperoxidase (MPO), nitric oxide (NO) in the cell.

Enzyme MPO is also an indirect indicator of neutophil infiltration and its levels measured at different time intervals induced no noticeable change but the lowest production was observed with imipenem treatment. In a similar study by Bansal and Chhiber (2010), mice induced with bacterial lung infection were treated with Augmentin® and curcumin (an immunomodulatory and anti-inflammatory agent) alone and in combination, the results showed that Augmentin® in combination with curcumin reduced the nitric oxide levels better than when Augmentin® was administered alone due to the antiflammatory properties of curcumin but curcumin administered alone had no effect on the lung homogenate. This suggests that though treatment with imipenem inhibit bacterial proliferation, adjunct therapy with antioxidative and anti-inflammatory properties may also be required (Mun et al., 2013; Betts and Wareham, 2014; Bali et al., 2016). This further confirms other studies on the significance of combined therapy in the treatment of ESBL infections (Iroha et al., 2010; Ikegbunam et al., 2014; Zhu et al., 2015).

NO generated during acute infection causes tissue damage by acting as a free radical and by generating more active species such as peroxynitrite which lead to lipid peroxidation of the cell membrane (Ward, 2010) and resulted in acute lung injury (Matthay et al. , 2012; Zhao et al., 2017). The level of cellular injury which might have occurred due to release of reactive oxygen species as a result of lipid peroxidation was also estimated in terms of MDA levels in lung homogenates. Treatment of mice with imipenem alone in the present study also resulted in decreased MDA levels in infected lung tissue thereby reducing lung injury. The results of the present study clearly demonstrate the positive effect of imipenem as reduced acute lung injury associated with pneumonia was observed in treated animals due to decreased production of various inflammatory markers such as NO, MPO and MDA and neutrophil recruitment. The P- values for MDA and NO was .030 and 0.002, respectively.

Conclusion

Pulmonary inflammatory damage caused by induced infection with ESBL producing isolates was susceptible to treatment with imipenem. Although, the observations *in vitro* and in animal models are not always applicable in clinical practice which may be due to disparate findings and the vast genetic heterogeneity of the organism and because the studies and case series that illustrate the experience with different antibiotics in the treatment of ESBL infections are difficult to interpret and do not have a direct correlation with clinical experiences. But be that as it may, the use of the carbepenems as a monotherapy or in combination suffices for the treatment of infections caused by ESBL isolates after appropriate susceptibility testing.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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