African Journal of Microbiology Research

Volume 8 Number 42, 15 October, 2014
ISSN 1996-0808

Academic Journals

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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Vol. 8(42), pp. 3630-3638, 15 October, 2014
DOI: 10.5897/AJMR2014.6995
Article Number: 00BE6F048177
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Assessment of antimicrobial effect of moringa: *In vitro* and *in vivo* evaluation

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Received 5 July, 2014; Accepted 6 October, 2014

This study was carried out to evaluate the antibacterial activity of water and ethanol extracts of *Moringa oleifera* (lam) leaves against major food poisoning reference strains using agar well diffusion method. Ethanolic extract showed greater antibacterial activity than water extract. The highest zones of inhibition were against Gram negative bacteria: *Escherichia coli* O157 ATTC 700728, *E. coli* O78, *E. coli* O26, *Salmonella* Typhimurium ATCC 13311 and *Shigella bodyii* ATCC 9207 with 25, 20, 12, 12 and 11 mm, mean zones of inhibition respectively. Followed by Gram positive bacteria: *Staphylococcus aureus* NCINB 50080 and MRSA ATCC 13565 with 18 and 11 mm mean zones of inhibition. Against *E. coli* O157, ethanolic extracts was more effective than oxytetracycline. *In vivo* study was carried out on 36 growing New Zealand White (NZW*) rabbit fed 0, 0.15, 0.30 and 0.45% *M. oleifera* dry leaves, the rabbit's weight gain, average daily gain (*ADG) and feed conversion efficiency were higher for rabbits fed 0.15 (R_2) and 0.30% (R_3) Moringa than others. Faecal bacterial count revealed that groups R_3 and R_2 showed significantly lower *E. coli*, *S. aureus* and heterotrophic plate count and non-detectable limits of Salmonella than the other groups. Conclusion enhanced supplementations of dry *Moringa* leaves with concentration of 0.15 and 0.30%.

Key words: Moringa leaves, antibacterial, agar well diffusion, bacterial count, rabbit.

INTRODUCTION

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide (Al-Bari et al., 2006). The increasing prevalence of multi-drug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raised the specter of 'untreatable' bacterial infections and add urgency to the search for new infection-fighting strategies (Zy et al., 2005; Rojas et al., 2006). For a long time, plants have been an important source of natural products for health. The antimicrobial properties of plants have been investigated by a number of studies worldwide and many of them have been used as therapeutic alternatives because of their antimicrobial properties (Adriana et al., 2007). Plants have many antimicrobial properties as secondary metabolites such as alkaloids, phenolic compounds, etc. Plants are the cheapest and safest

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alternative sources of antimicrobials (Sharif and Banik, 2006; Doughari et al., 2007a). *Moringa oleifera* (Lam) is the most widely cultivated species of a monogeneric family, which is widely used for treating bacterial infection, fungal infection (Fahey, 2005). *M. oleifera* is one of the 14 species of family Moringaceae, native to India, Africa, Arabia, South Asia, South America and the pacific and Caribbean Islands (Julia, 2008). *Moringa* leaves contain phytochemical compounds which are considered full of medicinal properties (Monica et al., 2010). The *Moringa* plant provides a rich and rare combination of zeatin, quercetin, campfire and many other phytochemicals. It is very important for its medicinal value. Other important medicinal properties of the plant include antispasmodic (Caceres et al., 1992), diuretic (Morton, 1991), antihypertensive (Dahot, 1988), cholesterol lowering (Mehta et al., 2003), antioxidant, antidiabetic, hepatoprotective (Ruckmani et al., 1998), antibacterial and antifungal activities (Nickon et al., 2003). According to World Health Organization (WHO, 2002) and Andy et al. (2008) more than 80% of the world's population rely on traditional medicines for their primary health care needs. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds.

Much research has been done worldwide to identify and study antibacterial compounds found in medicinal plants (Lugman et al., 2007; Silva et al., 2008; Mbosso et al., 2010). According to Rios and Recio (2005) studies, using essential oils or isolated compounds such as alkaloids, flavenoids, sesquiterpenes, lactones, diterpenes, triterpenes and naphtoquinones to test antibacterial effects are necessary to validate the use of a range of popular medicines. The Moringa tree (*M. oleifera*), a phanerogamous plant native to India, has been the object of extensive study due to its multiple uses as raw material in the production of oils, foods, condiments and drugs (Makkar and Becker, 1997). Studies on this plant have revealed promising anti-inflammatory (Ezeamuzie et al., 1996), antifungal (Chuang et al., 2007) and antibacterial (Doughari et al., 2007b) properties. The latter has been attributed to different parts of the plant, such as the leaves, roots, seeds, flowers, frupeel and unripe pods (Anwar et al., 2007).

This study was undertaken to investigate *in vitro*, the potency of aqueous and ethanol extracts of *M. oleifera* (Lam) leaves as antimicrobial agent against some pathogenic bacteria: *Escherichia coli (E. coli)* O26, *E. coli* O78, *E. coli* O157, *Staphylococcus aureus* methicillin resistant *S. aureus* (MRSA) and *Salmonella* Typhimurium. Also, *in vivo* dry powder of *M. oleifera* Lam was studied. The leaves were evaluated as growth promoters and antimicrobial agent against some pathogenic bacteria, including aerobic plate count, *E. coli*, *S. aureus* and *Salmonella*.

MATERIALS AND METHODS

First experiment

Plant collection and preparation

M. oleifera leaves were collected from a private farm of a sandy soil cultivated with Moringa shrubs over an area of 10 feddans (Feddan = 4200 m^2) located in Nubaria province (180 km northern Cairo city). The leaves were washed properly and oven dried at 40°C until the moisture content reached 10-12%. Dry leaves were finally ground using BRAUN house type grinder, sieved (1 mm mesh) and the powder was kept in a well tight polyethylene bags at room temperature until further use.

Preparation of Moringa extracts

The dried powder samples of *Moringa* leaves were extracted with 100% ethanol and distilled water. The extracts were collected three times and filtered through Whatman number 1 filter paper and then concentrated on a rotary evaporator at 45°C as described by Lugman et al. (2011). The sample and solvent mass ratio was 1:2 during extraction. Dry extracts were kept at 4°C till when needed for the assay.

Total phenolic compounds in the extracts was estimated using the Folin Ciocalteu's phenol reagent, where galic was used as a standard. The total carotenoids were spectrophotometrically estimated at 450 nm according to AOAC (1995). For the HPLC, finger print analysis of phenolic compounds (%) using Shimadzu system (Shimadzu Corp. Kyoto, Japan) consisting of the LC-IOAD pumps and injection volume of 20 µl was applied, whereas the separation of compounds was monitored at 280 and 320 nm. The determination of the antioxidative capacity was performed by the reaction of antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide (H_2O_2). The residual H_2O_2 was estimated colorimetrically using Bio-diagnostic Kit according to Blois (2002).

Antimicrobial assay

Preparation of bacterial suspensions

Antibacterial activities were carried out against seven highly pathogenic foodborne pathogenic strains of animal origin, including two Gram positive bacterial reference strains; *S. aureus* (NCINB 50080) and MRSA (ATCC 13565) and five Gram negative bacterial reference strains, including *Salmonella* Typhimurium (ATCC 13311), *S. bodyii* ATCC 9207, *E. coli* O157 (ATTC 700728), *E. coli* O26 and *E. coli* O78 (poultry isolated). Agar well diffusion test (qualitative method). Suspension of bacterial strains was freshly prepared by inoculating fresh stock culture from each strain into separate broth tubes, each containing 7 ml of Muller Hinton Broth. The inoculated tubes were incubated at 37°C for 24 h. Serial dilutions were carried out for each strain, dilution matching with 0.5 Mc-Farland scale standard was selected for screening of antimicrobial activities. Oxytetracycline 30 µg/ml and Vancomycin 30 µg/ml with MRSA was used as reference drugs.

Agar well diffusion method

The antimicrobial activity of water and ethanolic extract of Moringa

against bacterial strains were evaluated by using the agar-well diffusion method of Katircioglu and Mercan (2006). Hundred mirolitres (µl) of cell culture suspension matching with 0.5 McFarland of target strains were spread onto the plates. For the investigation of the antibacterial activity, 50 µl of extracts, and antibiotic discs of reference drugs were added. Plates were left for 1 h at 25°C to allow a period of pre-incubation diffusion in order to minimize the effects of variation in time between the applications of different solutions. The plates were re-incubated at 37°C for 24 h. After incubation, plates were observed for antimicrobial activities by determining the diameters of the zones of inhibition for each of the strains. For an accurate analysis, tests were run in triplicate for each strain in order to avoid any error.

Second experiment

Rations and feeding experiment

Four batches of rabbit ration each of 100 kg were formulated to contain: 30% alfalfa hay, 25% ground yellow corn, 25% wheat bran, 14% soy bean meal (44%), 3% cane-molasses, 1.5% lime stone, 1% sodium chloride and 0.5% vitamins and mineral mixture (premix of multi vita company-Egypt). Moringa powder dry leaves were added, thoroughly mixed with feed ingredients of each batch at 0, 0.15, 0.30 and 0.45% for R_1 (control), R_2, R_3 and R_4, respectively. Experimental ration were pelleted at 0.3 cm diameter and packed in polyethylene bags until feeding.

Thirty six male growing New Zealand White (NZW) rabbits aged five weeks old, with an average body weight of 566.5 g was blocked by weight in four equal groups (nine animals each). Experimental rabbits were housed individually in galvanized metal wire cages equipped with feeding and water troughs. The first group of rabbits was fed on R_1, while the 2nd, 3rd and 4th groups fed on R_2, R_3 and R_4, respectively. Chemical composition of Moringa dry leaves and rabbits feed mixture is given in Table 4.

The feeding experiment was for 56 days, where animals were weekly weighed and feed consumption was individually recorded. During the last week of feeding experiment rectal samples of faeces were individually collected from animals of each group in sterilized sealed nylon bags, where faecal samples were subjected to microbiological examination.

Bacteriological analysis

General purpose nutrient media, enrichment media and other appropriate selective media (all obtained from Oxoid) were employed in the culturing and isolation of selected pathogenic bacteria in the study.

Preparation of media

All dehydrated media were prepared according to manufacturer's instructions. They were mixed with distilled water and dissolved by gentle heat to boil. The media were sterilized in an autoclave at 121°C for 15 min. The sterile media were dispensed or poured into sterilized Petri-dishes and allowed to cool. The sterility of the prepared media was checked by incubation of blindly selected plates at 37°C for 24 h.

Bacterial isolation, identification and enumeration

Ten grams of the fecal sample were weighted in a sterile stomacher bag, and then 90 ml from maximum recovery diluents was added to the sample, the sample was well mixed using stomacher machine. Further serial dilution was done if needed by means of a pipette. 1 ml of the diluent was transferred into two Petri dishes, and then the media were poured into the dishes and were incubated at 37°C for 24-48 h. After incubation period, the counting of the colonies was done using the Stuart Digital colony counter.

Enumeration of total plate count was carried out according to *****EN ISO 4833 (2003) (European Union of international standard 4833: 2003). Ten-fold serial dilution of the bacterial suspension was made. This was done until 10^{-7} dilution was achieved. 0.1 ml was then pipetted from the 10^{-7} dilution onto the surface of each of two Petri dishes containing 15 ml of a solidified and sterile plate count agar (PCA), and then spread evenly with a sterile glass spreader. The plates were then incubated for a maximum of 24 h (including the control plates).

Detection of *Salmonella* was carried out according to modified *ISO 6579 (2002) (International Standard 6579: 2002). Twenty five grams of the fecal sample were weighted in a sterile stomacher bag or flask, and then 225 ml of buffer peptone water was added then 1 ml was plated onto xylose lysine deoxycholate agar (*XLD) plates then incubated at 37°C for 18 h. Typical colonies of *Salmonella* in XLD were red with black center. Biochemical reaction (triple sugar iron agar, lysine iron agar, citrate agar and urea agar) was used for confirmation of *Salmonella* typical colonies.

Enumeration of *E. coli* was carried out according to ISO 16649-2 (2001), 5 ml of violet bile lactose agar medium was added and a 10 ml second layer of the same media was added after the solidification of the agar and then incubated at 44°C for one day. Suspected colonies were confirmed to be *E. coli* by IMViC tests.

Enumeration of coagulase positive *S. aureus* was carried out according to ISO 6888-1 (1999). The fecal sample was prepared as mentioned before and dilution was carried out to 10^{-8}. 1 ml of each dilution was plated onto Baird-Parker agar (Oxoid). Then the plates were incubated aerobically at 37°C/24 h. Black colonies with hallow zone around were confirmed by biochemical including catalase, oxidase, indole, methyl red, Voges Proskauer, Simmon's citrate, urease test, hydrogen sulfide production in triple sugar iron agar medium, sugar fermentation test using different sugars, arginine hydrolysis test, hippurate hydrolysis test, nitrate reduction test, coagulase test were carried out (Quinn et al., 2002).

Statistical analysis

Data of fecal bacterial count were subjected to one-way analysis of variance according to steel Steel and Torrie (1980) applying the general linear model procedure of Statistical Analysis System (*SAS) (2001). Duncan's multiple range tests (1955) was applied to separate significant means.

RESULTS AND DISCUSSION

In vitro antimicrobial activities of *Moringa* leave extracts

In this study, the ethanolic leaf extract of *M. oleifera* was found to produce respectively, the highest zones of inhibition (25, 20, 12, 12, 11 mm) against all the Gram negative bacteria (*E. coli* O157 ATTC 700728, *E. coli* O78 (poultry isolate), *E. coli* O26 (poultry isolate), *S.* Typhimurium ATCC 13311 and *S. bodyii* ATCC 9207). The zones of inhibition for the Gram positive bacteria, *S. aureus* NCINB 50080 and MRSA ATCC 13565 were 18 and 11 mm, respectively. Some zone of inhibition reach more than one and a half to twice more effective than

Table 1. Some phytochemical compounds and antioxidants capacity of water and ethanolic extracts of *Moringa* leaves.

Item	Water extract	Ethanolic extract
Total carbohydrates (%)	26.76±1.58	23.43±1.45
Total chlorophyll (g/kg)	6.54±0.34	7.54±0.65
Total carotenoids (mg/kg)	823±54	1567±151
Total phenols (mg/g)	6.01±0.14	41.35±0.47
Total flavonoids (mg/g)	17.61±0.09	22.56±0.59
Total antioxidant capacity (µg/ml)	0.17±0.01	0.35±0.02

Table 2. Chemical composition of *Moringa* dry leaves and rabbits feed mixture.

Item	Moringa leaves	Feed mixture
Moisture (%)	10.23	10.00
Dry matter composition (%)		
Crude protein	31.06	19.11
Crude fiber	9.81	8.29
Ether extract	11.34	2.76
Soluble carbohydrates	29.31	54.02
Ash	18.48	15.82

Chemical composition was determined according to A.O.A.C. methods (1995).

Table 3. *In vitro* evaluation of water and ethanolic extracts of *Moringa* on pathogenic microorganisms under study when compared with reference drugs.

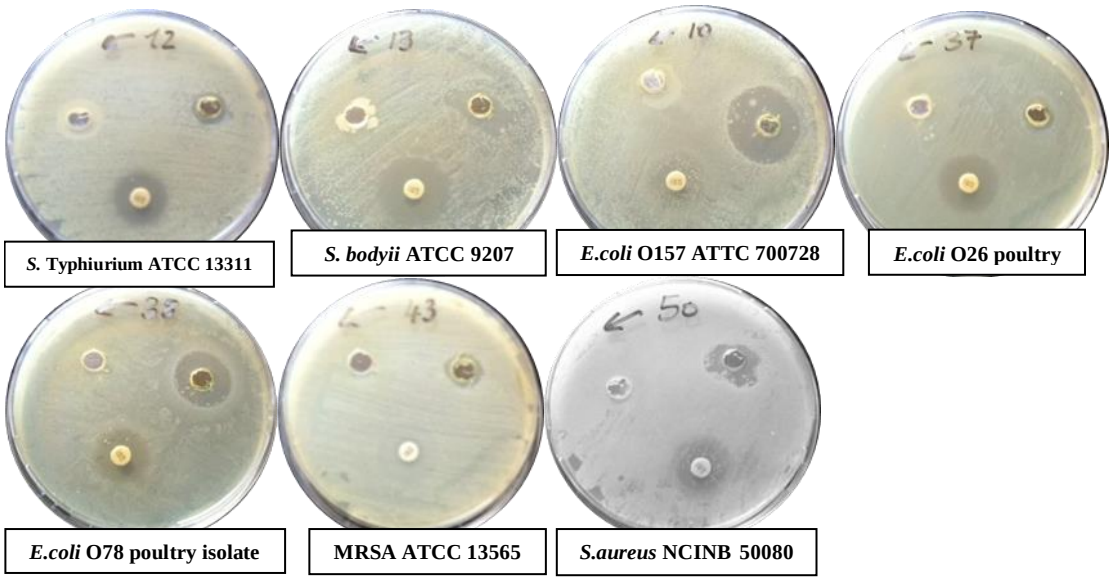
Strain	Aqueous extract	Ethanolic extract	Oxy-tetracycline OT 30 µg/d	Vancomycin VA 30 µg/d
Gram Negative Bacteria				
S. Typhimurium ATCC 13311	-ve	12	16	ND
S. bodyii ATCC 9207	-ve	11	22	ND
E. coli O157 ATTC 700728	-ve	25	13 static	ND
E. coli O26 (poultry isolate)	-ve	12	18	ND
E. coli O78 (poultry isolate)	-ve	20	16	ND
Gram Positive Bacteria				
MRSA ATCC 13565	-ve	11	ND	10/20s
S. aureus NCINB 50080	-ve	18	16	ND

reference antibiotic such as Oxytetracycline (30 µg/disc) as in the case of *E. coli* O157 ATTC 700728, where antibiotic showed zone of bacteriostatic effect reaching 13 mm diameter while *M. oleifera* ethanolic extracts show zone of inhibition reaching 25 mm. On the contrary, water extracts do not show any hindrance effect on the tested bacteria as shown in Table 3.

Antibacterial activity of water and ethanol extracts of *M. oleifera* Lam. leaves in Egypt were evaluated. Agar well diffusion method was applied to be used in this study. The ethanolic extract has greater antibacterial activity than the corresponding water extracts (Plates 1 to 7).

Growth performance

Body weight gain, average daily gain (ADG) and feed conversion efficiency of rabbits fed on experimental ration



Plates 1. Water extract, antibiotic reference drug and ethanolic extract in sequence anticlockwise with the arrow.

Table 4. Growth performance of rabbits fed rations with different supplements of *M.oleifera* dry leaves.

Item	Experimental rations				
	R_1 (0%)	R_2 (0.15%)	R_3 (0.30%)	R_4 (0.45%)	SEM
Animal No.	9	9	9	9	-
Duration period	---------- 56 days ----------				
Initial weight, g	560	573	571	562	24.07
Final weight, g	2024^b	2192^a	2219^a	1997^b	62.01
Body weight gain, g	1464	1619	1648	1435	64.66
Average daily gain, g	26.14^b	28.91^a	29.43^a	25.63^b	1.15
Dry matter intake, g	148^b	146^b	155^a	144^b	2.74
Feed Conversion (g. intake/g. gain)	5.66^c	5.05^a	5.27^b	5.62^c	0.11

a, b and c: Means with different superscripts in the same row are significantly different at ($P<0.05$) SEM=standard error of means.

are given in Table 4. Total weight gain and ADG of rabbits fed on 0.15 and 0.30% Moringa leaves ration were higher than those fed 0% (control) and 0.45% Moringa (R1 and R4) with no significant difference ($P<0.05$) between the two groups. A similar trend was observed for the feed conversion efficiency (g DM intake/g weight gain) where the rabbits fed on R2 and R3 diet performed better (5.05 and 5.27, respectively). Body weight gain of rabbits fed Moringa was 5.66 and 5.62, respectively. Body weight gain of rabbits fed Moringa supplemented rations relative to those fed control (R_1) was higher by 10.6, 12.5 and 2.0% with rations R_2, R_3 and R_4, respectively. Several previous studies reported that the inclusion of Moringa leaves in the diets of rabbits was associated with better weight gain (Nuhu, 2010; Adeniji and Lawal, 2012; Dougnon et al., 2012). None of these studies mentioned any adverse effect of Moringa leaves on the growth rate with increasing level of supplementation. However, Ewuola et al. (2012) found that rabbits that fed diets containing 0, 5, 10 and 15% Moringa (MOLM) has lower ADG with increasing Moringa substitution level being 6.8, 5.4, 6.5 and 3.8 with diets containing 0, 5, 10 and 15% Moringa leaves. There is no rigid explanation for the adverse effect of feeding o.45% Moringa ration on weight gain of rabbits in this study. It might hold true that, Moringa leaves which are known for their high contents of essential amino acids, vit A, B, C and E, antioxidant and antimicrobial compounds could play a role as a growth promoter at certain supplementation level. While its high contents of phenols,

Table 5. Pathogenic bacterial count in faecal samples of rabbits fed different *M. oleifera* leaves supplements.

Feeding groups	Heterotrophic plate count ($\times10^6$)	Salmonella ($\times10^4$)	*E. coli* ($\times10^5$)	*S. aureus* ($\times10^2$)
Control (0% M)	28.33±0.96[a]	2.56 ±0.57[b]	2.47±0.11[b]	30.00±1.63[a]
Group2 (0.15% M)	2.50±0.82[b]	ND	1.70±0.17[b]	1.83±0.09[b]
Group 3 (0.30% M)	1.47±0.19[b]	ND	ND	1.40±0.08[b]
Group 4 (0.45% M)	30.67±0.55[a]	5.67 ±1.64[a]	7.46±0.51[a]	28.00±0.82[a]

All samples were counted in 1 g faecal sample except *Salmonella* in 25 g sample. Each value is mean of two pooled faecal samples from each group. ND = non-detectable; a, b: means within the same raw with different superscripts are significantly different at ($p<0.05$).

tannins, alkaloids and cumarins might accumulate in the body, causing inhibition of feed utilization or decomposition of some antimicrobial compounds.

In vivo antimicrobial activities of *Moringa* extracts

Faecal bacterial count as shown in Table 5, revealed that group R_3 (0.30% M) and group R_2 (0.15% M) showed the least bacterial count, which was significantly lower than that in the other two groups; control group R_1 (0% M) and group R_4 (0.45% M). Groups R_2 and R_3 showed non-detectable limits of *Salmonella* when compared with control group R_1 and group R_4. *E. coli* count showed that group R_3 was free of *E. coli*, group R_2 count was $1.70\pm0.17\times10^5$ which was significantly lower than control group R_1 and group R_4 with count reaching $2.47\pm0.11\times10^5$ and $7.46\pm0.51\times10^5$, respectively. Groups R_2 and R_3 showed low *S. aureus* count of $1.83\pm0.09\times10^2$ and $1.40\pm0.08\times10^2$ respectively, which were significantly lower than the control group R_1 ($30.00\pm1.63\times10^2$) and group R_4 ($28.00\pm0.82\times10^2$). The same finding was observed in indicated heterotrophic plate count where groups R_2 and R_3 showed a low bacterial count; $2.50\pm0.82\times10^6$ and $1.47\pm0.19\times10^6$ respectively, which were significantly lower than the control group R_1 ($28.33\pm0.96\times10^6$) and group R_4 ($30.67\pm0.55\times10^6$).

Antibacterial activity of water and ethanol extracts of *M. oleifera* Lam. leaves in Egypt were evaluated. Agar well diffusion method was applied to be used in this study. The ethanolic extract has greater antibacterial activity than the corresponding water extracts (Plates 1 to 6).

In our investigation, highest zones of inhibition were found in the leaf ethanolic extract against Gram negative bacteria under investigation: *E. coli* O157 ATTC 700728, *E. coli* O78 (poultry isolate), *E. coli* O26 (poultry isolate), *S.* Typhimurium ATCC 13311 and *S. bodyii* ATCC 9207 with the zone of inhibition equals 25, 20, 12, 12, 11 mm, respectively.

Agar well diffusion test against Gram positive bacteria, including *S. aureus* NCINB 50080 and MRSA ATCC 13565 show zone of inhibition equals 18 and 11 mm, respectively. A zone of inhibition was more than one and a half to twice as much effective as known antibiotic oxytetracycline (30 µg/disc) as in the case of *E. coli* O157 ATTC 700728 w zone of bacteriostatic effect of 13 mm diameter while, *M. oleifera* ethanolic extracts show zone of inhibition reaching 25 mm. On the contrary, aqueous extract does not show any hindrance effect on the tested bacteria shown in Table 3 and Plate 1. Many authors have reported antimicrobial activities of plant extracts on food-borne pathogens (Moreira et al., 2005; Afolabi, 2007; Kotzekidou et al., 2007; Atiqur and Sun 2009), which indicates the possibility of use of plant extract as sanitizers and preservatives. The antimicrobial activity of the extracts tested, reveal bioactivity on wide range of pathogenic and toxigenic organisms liable to cause food-borne illnesses such as *E. coli, S. aureus, P. aeruginosa, S. typhi* and *S*. Typhimurium (Bukar et al., 2010).

This result is because in the traditional method of treating a bacterial infection, a decoction of the plant parts or boiling the plant in water is employed, whereas, according to the present study, preparing an extract with an organic solvent was shown to provide a better anti-bacterial activity, in accordance with the results obtained by Nair et al. (2005). Gram-negative bacteria have been found to be less susceptible to plant extracts in earlier studies done by other researchers (Kuhnt et al., 1994; Afolayan and Meyer, 1995). Vinoth et al. (2012) reported that the ethanolic extract was active against *S. typhi* and *S. aureus* whereas the aqueous extract exhibited an inhibitory effect on *S. aureus* only, and proved that the ethanol extract was more active than the aqueous extract against *S. typhi*.

Previous studies conducted by Rahman et al. (2009) showed that ethanol extract of fresh *M. oleifera* leaves has extensive antibacterial effect against all tested Gram-negative bacteria (*S. shinga, P. aeruginosa, S. sonnei, Pseudomonas* spp.) and some Gram-positive bacteria (*B. cereus, B. subtilis, S. lutea, B. megaterium*) and their respective zones of inhibition were 17.5±0.34, 21.21±0.05, 21.50±0.08, 21.25±0.1.3 and 16.25±0.04, 20.23±0.56, 19.50±0.21, 20.50±0.04 mm, respectively. Cáceres et al. (1991) found *M. oleifera* leaf extracts can inhibit the growth of *S. aureus* and *P. aeruginosa*. Likewise, in a study by Valsaraj et al. (1997) evaluating the

antibacterial effect of 78 plants used in India to treat infectious diseases, *P. aeruginosa* and *S. aureus* were inhibited by extracts of Moringa peel. Mandal and Mandal (2011), Paul et al. (2011), Prabhll et al. (2011) and Suarez et al. (2003) found Moringa extracts produced bacteriostatic and bactericidal effects on *S. aureus*. Djakalia et al. (2011) stated that *Moringa* leaf extract have antimicrobial activities. They inhibit the growth of *S. aureus* in the feed and rabbits intestines. Bukar et al. (2010) reported that *M. olifera* leaf ethanolic extract had the broadest spectrum of activity of the test bacteria. The results showed that activity against four bacterial isolates *Enterobacter* spp. (7 mm), *S. aureus* (8 mm), *P. aeroginosa,* (7 mm) and *E. coli* (7 mm) were sensitive at a concentration of 200 mg/ml. While Shigella spp. and *S.* Typhi were not sensitive at all concentrations used. Also, Napoleon et al. (2009) reported *Enterobactor* spp., *S. aureus, P. aeruginosa, S.* Typhi and *E. coli* to be sensitive to ethanol, chloroform and aqueous extract of *M. olifera* leaf at a concentration of 200 mg/l.

The results of the present work totally oppose the findings of investigations of many authors who observed resistance of *E. coli* to *Moringa* extract which matches findings from a study of the antibacterial properties of Indian plants showing Moringa extracts to be ineffective against *E. coli* (Bhawasar et al., 1965). Also, Rajendrhan et al. (1998) reported *E. coli* to be resistant to Moringa extracts.

On the other hand, the present study suggests that *M. oleifera* Lam. leaves used contain bio-components whose antibacterial potentials are highly comparable with that of the antibiotic Oxytetracycline against all Gram-negative and Gram-positive bacteria tested. The activity of the plant against both Gram-positive and Gram negative bacteria may be indicative of the presence of broad-spectrum antibiotic compounds in the plant (Siddhuraju and Becker, 2003; Vaghasiya and Chanda, 2007). Today, most pathogenic organisms are becoming resistant to antibiotics (Chandarana et al., 2005). Moringa leaves have been reported to be a good source of natural antioxidants such as ascorbic acid, flavonoids, phenolics and carotenoids (Dillard and German, 2000). To overcome this alarming problem, the discovery of novel active compounds against new targets is a matter of urgency. Thus, *M. oleifera* Lam. could become promising natural antimicrobial agents with potential applications in the pharmaceutical industry for controlling the pathogenic bacteria. Doughari et al. (2007b) observed inhibition halos up to 8 mm when challenging *Salmonella* with aqueous and ethanolic Moringa leaf extracts. The authors attributed the antibacterial effect to the presence of saponine, tannic, phenolic and alkaloid phyto constituents. However, if plant extracts are to be used for medicinal purposes, issues of safety and toxicity will always need to be considered.

Moringa roots have antibacterial activity (Rao et al., 2001) and are reported to be rich in antimicrobial agents. These are reported to contain an active antibiotic principle, pterygospermin, which has powerful antibacterial and fungicidal effects.

A similar compound is found to be responsible for the antibacterial and fungicidal effects of its flowers (Rao, 1957). The root extract also possesses antimicrobial activity attributed to the presence of 4- α-L-rhamnosyloxybenzyl isothiocyanate (Eilert et al., 1981). The aglycone of deoxy-niazimicine [N-benzyl, S-ethyl thioformate] isolated from the chloroform fraction of an ethanol extract of the root bark was found to be responsible for the antibacterial and antifungal activities (Nickon et al., 2003). The fresh leaf juice was found to inhibit the growth of microorganisms, *P. aeruginosa* and *S. aureus* (Caceres et al., 1992).

Conclusion

In conclusion, ethanolic *Moringa* leaves extract were capable of inhibiting the growth of Gram- negative and positive bacteria. *M. oleifera* leaves at concentration of 0.15-0.30% can treat common pathogenic bacterial infections and could be used for preventing and treating malnutrition and to enhance growth rate. There is need to carry out more pharmacological studies to support the use of *M. oleifera* as a medicinal plant.

Conflict of interest

The author(s) have not declared any conflict of interests.

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Vol. 8(42), pp. 3639-3649, 15 October, 2014
DOI: 10.5897/AJMR2014.6892
Article Number: 171DC5648181
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Metal and antibiotic resistance among heterotrophic bacteria inhabiting hospital waste water and polluted sea water

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Received 8 May, 2014; Accepted 12 September, 2014

The pollution of marine environment with heavy metals, antibiotics, dyes and surfactants may possess a serious threat to human life and the other organisms because of their toxicity and ability to persist for long time. The metals and antibiotics resistance of 48 heterotrophic bacterial isolates from hospital waste water and polluted sea water were studied. The minimum inhibitory concentrations (MICs) of heavy metals were different for each isolate. All isolates showed high resistance to nickel, chromium, barium and copper. On the other hand, mercury was the highest toxic metal against all the isolates. The frequencies of resistance for all isolates to each metal ion tested were as follows: Ni, 88%; Ba, 88%; Cr, 75%; Cu, 38% and Hg, 13% for hospital waste water isolates, and Ni, 88%; Ba, 75%; Cr, 50%; Cu, 50% and Hg, 0% for seawater bacteria. The response of the isolates to five antibiotics was tested; it ranged from complete resistance to total sensitivity. The hospital waste water isolates showed resistance for 4 of 5 antibiotics with percentages that ranged from 13 to 100%. On the other hand, 88% of seawater isolates resisted only one antibiotic. Multiple-metal resistance was exhibited by 100% of heterotrophic bacteria, while penta-metal-resistant bacteria was observed only in hospital waste water isolates. Multiple-antibiotic resistance was exhibited by 63% of the hospital waste water bacteria. The highest incidence of metal-antibiotic double resistance was observed in hospital waste water isolates compared with sea water isolates. Moreover, all isolates exhibited multiple resistances for different dyes as crystal violet, iodine and sufranine and other chemicals such as sodium lurayl sulfate. The bacterial isolates from hospital waste water showed higher resistance to dyes and surfactant than those isolated from polluted seawater. The composition of bacterial communities which were resistant to different chemicals ranged from 0.02 to 0.33% for the bacterial pathogens and from 1.95 to 15.0% for the fecal bacteria in the hospital waste water whereas, in seawater samples, the percentages of bacterial pathogens ranged from 0.04 to 0.12% and the percentages fecal bacteria ranged from 0.95 to 29.10%. This finding suggests that the discharge of hospital waste water without preliminary treatment is an important source for the spread of new phenotype bacteria with multiple-resistance in natural habitats which can pose a public health risk.

Key words: Metal resistance, antibiotic resistance, heterotrophic bacteria, hospital waste water, polluted sea water.

INTRODUCTION

The problem of hospital waste is becoming increasingly important. These effluents represent a major threat to the receiving environment and to human health. Scientists wonder about the long term effects they can have on

human health and the environment; taken in consideration the nature and significance of specific substances they contain (Acharya and Singh, 2000; Kümmerer, 2001; Askarian et al., 2004). From a microbiological view, these effluents contain microbes' specific to the hospital medium. These microbes are often polyresistant to antibiotics and responsible for many infections (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, etc...) and fecal coliform bacteria (*Escherichia sp.* and fecal *streptococci*) (Ibekwe et al., 2003; Ivnitsky et al., 2007). From the chemical point of view, the hospital effluents contain organic compounds, heavy metals such as mercury, silver, nickel, chromium and cobalt (Acharya and Singh, 2000; Kümmerer, 2001; Mandal and Dutta, 2009; Mathur et al., 2012). Also, effluents contain dyes that serve as principal source of water pollution (Hassan et al., 2013). Most of these dyes are potentially toxic to aquatic Life. Furthermore, color of the dyestuff interrupts the aquatic environment (Khadijah et al., 2009; Hassan et al., 2013). Pollutants discharges in natural environments may alter the natural composition of microbial communities resulting in poor taxonomical diversity and allowing the survival of species resistant or able to degrade the pollutants. In chronically polluted environments microbial communities can acquire specific traits (such as heavy metal or antibiotic resistance), but in many cases these changes cannot be detected easily because they are sometimes unapparent. Complex adaptative mechanisms are initiated in microbial cell during the chronic exposure to pollutants that results in specific and discernable traits. Appearance of these traits is dependent on the presence of unfavorable factors for a long period of time necessary in stabilizing the metabolic pathways involved in conversion of pollutants (Aonofriesei, 2003). Much of the antibiotic used in humans and animals remains un-metabolized and thus a significant amount is added to the environment via excretion. This ultimately contributes to the residues of antibiotics in recipient waters. Antibiotics might also be added to the environment from pharmaceutical plants and as a result of the dumping of unused antibiotics. Also, antibiotics are entering the aquatic environment through hospital effluent. Besides antibiotics, resistant bacteria also enter into the aquatic environment (Diwan et al., 2010).

Until recently, the majority of study has been carried out on clinical material and thus, little is known about bacterial resistance to antibiotics in the natural environment. Hence, the role of antibiotic substances secreted into the natural environment has not been recognized in a comprehensive way and ever since has been one of the most controversial issues of microorganisms' ecology (Mudryk, 2002). Antibiotic resistant organisms are selected in the natural environments not only in presence of antibiotics but also in presence of some non-antibiotic substances including heavy metals. Genes conferring antibiotic resistance and genes conferring heavy metal resistance are most often found to be located on the same plasmid. That is why, if heavy metals are present in an environment as pollutant, many bacteria that survive in presence of them are found to be resistant not only to heavy metals but also to antibiotics. Thus antibiotic resistant organisms are selected to flourish even in absence of antibiotics.

A vast body of information is available in the literature on co-occurrence of antibiotic and heavy metal resistance in various types of natural isolates of bacteria (Chattopadhyay and Grossart, 2011). It has been for a long time; heavy metal pollution has become one of the most serious environmental problems. The pollution of environment with toxic heavy metals is spreading throughout the world along with industrial progress. So, presence of heavy metals even in traces is toxic and detrimental to all living organisms (Pandit et al., 2013). Presence of these heavy metals in the marine environment may pose a serious threat to the environment because of their ability to persist for several decades (Kamala-Kannan and Lee, 2008; Matyar, 2012). The main threats to human health from heavy metals are associated with exposure to lead, cadmium, chromium, mercury and arsenic (Nithya et al., 2011; Kacar and Kocyigit, 2013). Some metals were toxic often at low concentration, and microorganisms were influenced by this toxicity, that concerns their diversity and activities and consequently their sustainability (Karbasizaed et al., 2003; Habi and Daba, 2009). Microorganisms play an important role in the environmental fate of toxic heavy metals with a multiplicity of mechanisms (Chatterjee et al., 2014).

The present study aimed to investigate the heavy metal and antibiotic resistance in heterotrophic bacterial community inhabiting the hospital waste water and polluted seawater. The study extended to investigate the multiple resistance of these bacteria, and they resistance to other inhibitors such as dyes and surfactant.

MATERIALS AND METHODS

Site description

The Eastern Harbor of Alexandria (Egypt), selected as the study area, is a semi-enclosed, protected and shallow bay (2-12 m). The circular basin covers an area of about 2.8 km^2 and occupies the central part of Alexandria coast. One major and 11 minor outfalls discharge large amounts (exceeding than 230000 m^3d^{-1}) of untreated waste waters into the Eastern Harbor water (Sabry et al., 1997). Poorly untreated industrial waste, domestic sewage, shipping industry and agricultural runoff are being released to the coast (Frihy et al., 1996).

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Table 1. Minimal inhibitory concentrations of various heavy metals (ppm) to the heterotrophic bacterial isolates.

Bacterial isolate	Metal ions (ppm*)				
	Hg	Ni	Cr	Ba	Cu
Waste water					
H1	1	30	45	45	5
H2	1	10	50	45	40
H3	10	70	55	50	5
H4	1	30	40	50	5
H5	1	30	40	40	5
H6	1	30	40	40	5
H7	1	30	40	40	40
H8	1	30	60	45	40
Sea water					
S1	1	30	50	45	5
S2	1	10	40	40	5
S3	1	10	40	45	40
S4	1	40	60	50	40
S5	1	30	40	40	20
S6	1	30	45	45	20
S7	1	30	45	45	20
S8	1	5	40	40	20

Samples collections

A total of 48 water samples were collected during Autumn 2013.Eight samples were collected weekly in triplicate from the hospital waste water discharge (H), El-Meery Hospital, Egypt. Other eight sea water samples (S) were collected weekly in triplicate from Alexandria Eastern Harbor, Egypt. Sampling was performed according to WHO (1995) and Clesceri et al. (2012).

Bacteriological examination

Total viable heterotrophic bacterial counts (THVC) were detected on Zobell agar medium (Oxoid LTD, England). Decimal dilutions of water samples were prepared and the pour plate technique was used (Clesceri et al., 2012).

Staphylococcus sp, and *Vibrio* ssp. were detected on manitol salt agar and thiosulphate citrate bile salt sucrose agar (TCBS), (Oxoid LTD, England), respectively (Atlas, 1997). Total coliforms (TC), *Escherichia coli* (EC) and fecal streptococci (FS) were enumerated on m-endoles agar medium, mFC agar and m-enterococcus agar medium, (Difco, Detroit, MI), respectively. The membrane filtration technique was used according to ISO 7899/2 (1984) and ISO 9308/1 (1990). Three replicates for each sample were used and the final counts were estimated as colony forming units (CFU/100 ml).

Heavy metals toxicity test

To examine the ability of heterotrophic bacterial isolates to resist heavy metal, cells of overnight grown cultures (10^7 CFU/ml) were inoculated on nutrient agar plates supplemented with different concentrations (10-700 ppm) of heavy metals (Sterilized stock solutions of each metal salt: $HgCl_2$, $NiSO_4$, $K_2Cr_2O_7$, $BaCl_2$ and $CuSO_4$ were used). Cultures were incubated at 30°C for 24-48 h and the cell growth was observed. Plates containing media without metal were inoculated in the same way to act as controls (Hookoom and Puchooa, 2013).

Antibiotics resistance test

Resistance of heterotrophic bacterial isolates from water samples were tested against different antibiotics by the disc diffusion method according to Bauer et al. (1966). The following five antibiotics were tested with their amounts: levofloxacin (25 mg), ceftriaxone (20 mg), vancomycin (5 mg), imipenem (30 mg) and amikacin (30 mg). The antibiotic discs (Oxoid Company, England) were applied to the surface of the seeded medium. The degree of resistance of the bacterial isolates was determined on the basis of the measurements of lightened zones (mm) around the disc. However, the multiple antibiotic resistances (MAR) index of isolates against tested antibiotics was calculated based on the following formula: MAR index = X / (Y x Z); X is the total of bacteria resistant to antibiotics; Y is the total of antibiotics used in the study; while Z is the total of isolates (Lee et al., 2009).

Dyes and surfactant resistance test

The ability heterotrophic bacterial isolates to grow in the presence of dyes (crystal violet, iodine and sufranine) at different concentrations (0.001, 0.01, 0.1 and 1%); and surfactant (sodium lurayl sulfate, SLS) at concentrations 50, 100, 200, 300, 500 and 700 ppm were investigated, individually. Cells of overnight grown cultures (10^7 CFU/ml) were inoculated on nutrient agar plates using pour technique method. Surfactant and dye stock solutions were prepared and sterilizes. A range of potential inhibitors at diagnostic concentrations were added to nutrient agar medium after sterilization. The growth was detected after incubation at 30°C for 24-48 h. Plates containing media without inhibitors were inoculated in the same way to act as controls (Williams et al., 1989).

RESULTS

Response to heavy metals

The minimum inhibitory concentrations (MICs) of heavy metals were different for each isolate. All isolates showed high resistance to Ni, Cr, Ba and Cu. On the other hand, mercury was the highest toxic metal against all the isolates; they showed 100% no growth in 1 ppm concentration for sea water isolates and 88% of waste water isolates exhibited no growth at the same concentration. The toxic effects of these metals increased with increasing concentration. The bacterial isolate (H3) from hospital waste water exhibited the highest resistance to most metal tested, Its MIC reached 70, 55, 50, 40 and 10 ppm for Ni, Cr, Ba, Cu and Hg, respectively . The isolate (S2) from sea water was the highest sensitive to metals; it exhibited absence of growth in 40, 40, 10, 5 and 1 ppm of Cr, Ba, Ni, Cu and Hg, respectively (Table 1).

The frequencies of resistance for all isolates to each metal ion tested were as follows: Ni, 88%; Ba, 88%; Cr, 75%; Cu, 38% and Hg, 13% for hospital waste water isolates, and Ni, 88%; Ba, 75%; Cr, 50%; Cu, 50% and Hg, 0% for seawater bacteria (Table 2).

Multiple metals resistance

Multiple metals resistance (MMR) is presented in Table 3.

Table 2. The frequencies of the heterotrophic bacterial isolates resistant to various concentrations of metals ions.

Metal ion	Cumulative % of isolates resistant at the following concentrations (ppm)								
	1	3	5	10	20	30	40	50	70
Waste water									
Hg	13	13	13	13	0	0	0	0	0
Ni	100	100	100	88	88	13	13	13	0
Cr	100	88	75	75	75	63	50	38	0
Ba	100	100	100	100	88	88	75	25	0
Cu	63	50	38	38	38	13	0	0	0
Sea water									
Hg	0	0	0	0	0	0	0	0	0
Ni	100	88	88	63	63	0	0	0	0
Cr	100	100	100	100	88	50	50	25	0
Ba	100	100	100	100	75	75	75	25	0
Cu	75	63	50	50	25	25	0	0	0

Table 3. The multiple metal resistances of the heterotrophic bacteria inhabiting the hospital waste water and sea water.

Bacterial isolate	No. of metals to which isolates were resistant at different concentrations (ppm)							
	1	5	10	20	30	40	50	70
Waste water								
H1	3	3	3	3	2	2	1	0
H2	4	4	3	3	3	2	1	0
H3	5	4	4	3	3	3	2	0
H4	4	3	3	3	2	1	1	0
H5	4	3	3	3	0	0	0	0
H6	3	3	3	2	0	0	0	0
H7	4	4	4	4	1	1	0	0
H8	4	4	4	4	2	2	1	0
Sea water								
S1	4	3	3	3	2	2	1	0
S2	4	4	2	1	0	0	0	0
S3	4	4	3	3	3	1	0	0
S4	4	4	4	4	3	2	1	0
S5	3	3	3	1	0	0	0	0
S6	4	4	4	3	2	2	1	0
S7	4	4	4	3	2	2	1	0
S8	3	2	2	2	2	1	0	0

All bacterial isolates were resistant to more than one metal ions (multiple metals resistances) at 1, 5 and 10 ppm of metal ions. The bacterial isolates H3 were resistant to the five metals ions tested (penta-metal-resistant); these penta-metal-resistant bacteria (represented 13%) were exhibited only in hospital waste water isolates. At 1 ppm, 63% of hospital waste water isolates were tertra-metal-resistant and 25% of isolates were Tri-metal-resistant. Sea water isolates were either tertra-metal-resistant (75%) or tri-metal-resistant (25%). At 50 ppm, only one isolates (H3) from waste water were double-resistant and 50% of heterotrophic bacterial isolates were mono-resistant.

Antibiotics resistance

The resistance of heterotrophic bacteria isolated from sea water and hospital waste water samples against selected antibiotics was examined. As shown in Table 4, the patterns

Table 4. Inhibition zones (mm) of bacterial isolates against selected antibiotics.

Bacterial isolate	Inhibition zones* (mm)				
	Levofloxacin (25 mg)	Ceftriaxone (20 mg)	Vancomycin (5 mg)	Imipenem (30 mg)	Amikacin (30 mg)
Waste water					
H1	22±0.92	0	0	38±0.34	12±0.76
H2	8±0.60	0	8±0.25	28±0.90	9±0.08
H3	0	0	15±0.18	25±0.16	10±0.15
H4	0	0	21±0.15	17±0.87	0
H5	16±0.78	0	0	30±0.42	8±0.15
H6	8±0.24	0	10±0.32	24±0.70	9±0.20
H7	8±0.32	0	8±0.70	10±0.64	8±0.15
H8	0	0	0	15±0.18	11±0.15
Sea water					
S1	23±0.90	19±0.15	0	30±0.68	10±0.22
S2	25±0.62	19±0.25	0	43±0.90	13±0.19
S3	11±0.52	10±0.47	0	36±0.26	10±0.18
S4	30±0.42	15±0.18	0	42±1.09	18±0.35
S5	49±1.06	40±0.80	0	30±0.3 5	21±0.65
S6	9±0.28	15±0.16	0	26±0.75	9±0.35
S7	17±0.52	13±0.28	0	38±0.45	11±0.08
S8	26±0.30	49±0.35	12±0.24	31±0.28	34±0.40

*Values are average ± standard deviation (n=3).

of resistances of bacteria to various antibiotics were considerably differed. Bacteria inhabiting hospital waste water were more resistant to ceftriaxone, at the same time 100, 88, 63 and 63% of isolates were sensitive to imipenem, amikacin, levofloxacin and vancomycin, respectively with different degrees. The maximum range of inhibition zones were exhibited toward imipenem (10-38 mm).

On the other hand, isolates from seawater were resistant to vancomycin (except S8) and sensitive to other antibiotics with different degrees. The maximum ranges of inhibition zones were exhibited toward levofloxacin (9-49 mm) and ceftriaxone (10-49 mm) and the minimum range of inhibition zones were toward amikacin (9-34 mm).

The heterotrophic bacteria inhabiting hospital waste water were resistant to all antibiotics with different percentages (ranged from 13 to 100%), except imipenem that inhibited all isolates from hospital waste water. On the other hand, the heterotrophic bacteria inhabiting seawater were sensitive to all tested antibiotics except vancomycin.

Multiple antibiotics resistance

The percentage of multiple antibiotics resistances (MAR) were calculated. As shown in Figure 1, all heterotrophic bacteria inhabiting hospital waste water were resistant to at least one antibiotic; the majority showed resistance to two or three of the five tested antibiotics, then the percentages of MAR ranged from 20 to 60%. Most bacterial isolates from sea water resisted one antibiotic only, then the percentage of resistance frequencies to antibiotics did not exceed than 20%.

The multiple antibiotics resistance index of bacterial communities inhabiting hospital waste water was 0.38 while the MAR index for bacterial communities inhabiting sea water was 0.18. The MAR indexes indicated that there was a difference between the heterotrophic bacteria inhabiting hospital waste water and sea water in their resistance to the antibiotics used in this study.

Correlation between antibiotics and metals resistance

The percentage of metal and antibiotic double-resistant isolates was calculated by dividing the number of isolates resistant to a certain antibiotic and simultaneously to a specific metal ion by the total number of isolates resistant to this particular antibiotic x 100 and is represented in Table 5. The highest incidence (100%) of metal-antibiotic double resistance in waste water isolates exists between: nickel and ceftriaxone, vancomycine and amikacin; chromium and levofloxancin and amikacin; and finally barium and all antibiotic except imipenem. Moreover, the double resistance among seawater isolates exists between nickel, chromium and barium and only one antibiotic (vacomycin).

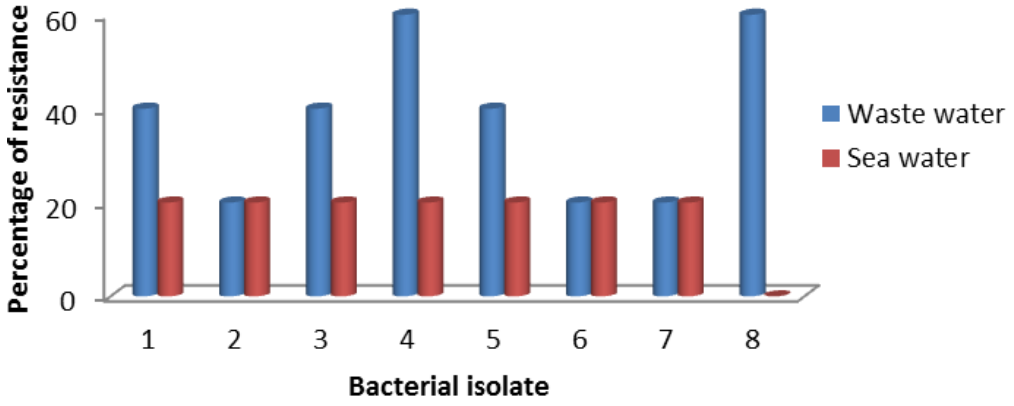

Figure 1. The percentage of multiple antibiotic resistances of bacterial isolates from hospital waste water and sea water.

Table 5. Correlation between antibiotic and metal resistance.

Antibiotic	Mercury				Nickel			Chromium			Barium			Copper		
	TNo.	No.	%	T%	No.	%	T%	No.	%	T%	No.	%	T%	No.	%	T%
Waste water																
Levofloxacin	3	1	33	13	2	67	25	3	100	38	3	100	38	1	34	13
Ceftriaxone	8	1	13	13	8	100	100	6	75	75	8	100	100	3	38	38
Vancomycin	3	0	0	0	3	100	38	2	67	25	3	100	38	1	34	34
Imipenem	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amikacin	1	0	0	0	1	100	13	1	100	13	1	100	13	0	0	0
Sea water																
Levofloxacin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ceftriaxone	0	0	0		0	0	0	0	0	0	0	0	0	0		0
Vancomycin	7	0	0	0	7	100	88	7	100	88	7	100	88	3	43	38
Imipenem	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amikacin	0	0	0	0	0	0	0	0	0	0	0		0	0		0

TNo, Total number of isolates resistant to the particular antibiotic; No., number of isolates resistant to metal and antibiotic; %, percent of total number of isolates resistant to the particular antibiotic; T%, per cent of total number of isolates (8 for waste water and 8 for sea water).

Resistance to different dyes

The average counts of bacterial isolates from hospital waste water showed higher resistance to crystal violet and iodine than those isolated from polluted seawater. The order of resistance to the dyes was found to be as crystal violet > iodine > sufranine in hospital waste water. The order of dyes resistance was found to be crystal violet > sufranine > iodine in seawater (Figure 2).

Multiple dyes resistance

Figure 3 shows that the heterotrophic bacteria isolated from hospital waste water and sea water resist the three dyes tested (the percentage of multiple dye resistance was 100%), except isolates H5 and H6 from hospital discharge water resist only two dyes (resist 66% of dyes). As well also, isolates 2S and 5S from seawater resisted only 33% of dyes.

Resistance to SLS surfactant

All the isolates were highly resistant to surfactant. Bacterial isolates from hospital waste water (H2, H3, and H4) and sea water (S2, S3 and S4) showed no growth in 200, 200 and >700 ppm of surfactant, respectively. Two bacterial isolates (H4 and H8) from hospital waste water were capable of tolerating higher concentration of

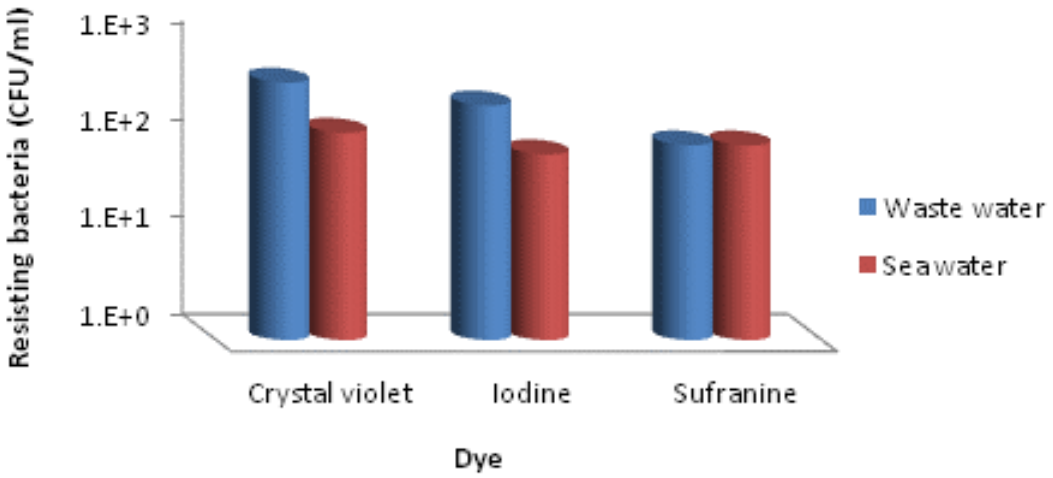

Figure 2. The average counts of heterotrophic bacteria resisting to 1% of different dyes, individually, in hospital waste water and sea water (control, 10^7 CFU/ml).

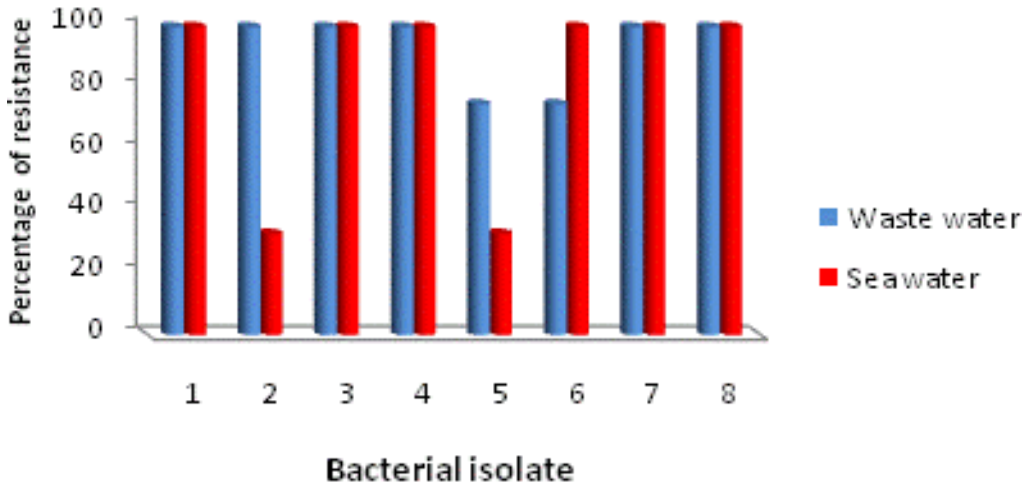

Figure 3. The percentage of dye resistance (at 1% of each dye, individually) of heterotrophic bacterial isolated from hospital waste water and sea water.

Table 6. Minimum inhibitory concentrations of SLS surfactant (ppm).

Waste water		Seawater	
isolate	**MIC**	**Isolate**	**MIC**
H1	200	S1	500
H2	200	S2	200
H3	200	S3	200
H4	> 700	S4	> 700
H5	100	S5	200
H6	200	S6	700
H7	500	S7	300
H8	> 700	S8	300

MIC, Minimum inhibitory concentrations.

surfactant more than 700 ppm compared to one bacterial isolates (S4) from sea water (Table 6).

Composition of the bacterial community

The variation in densities of pathogens and coliforms populations in hospital waste water samples was conducted. *Staphylococcus* sp. and *Vibrio* sp. were detected in all water samples collected during the period of study from the first to the eighth week. *Staphylococcus* sp. present in low counts ranged from $0.7x10^1$ to $4.7x10^1$ CFU/100 ml. *Vibrio* sp. ranged from $1.4x10^1$ to $7.3x10^1$ CFU/100ml (Figure 4a).

Hospital waste water samples had high densities of

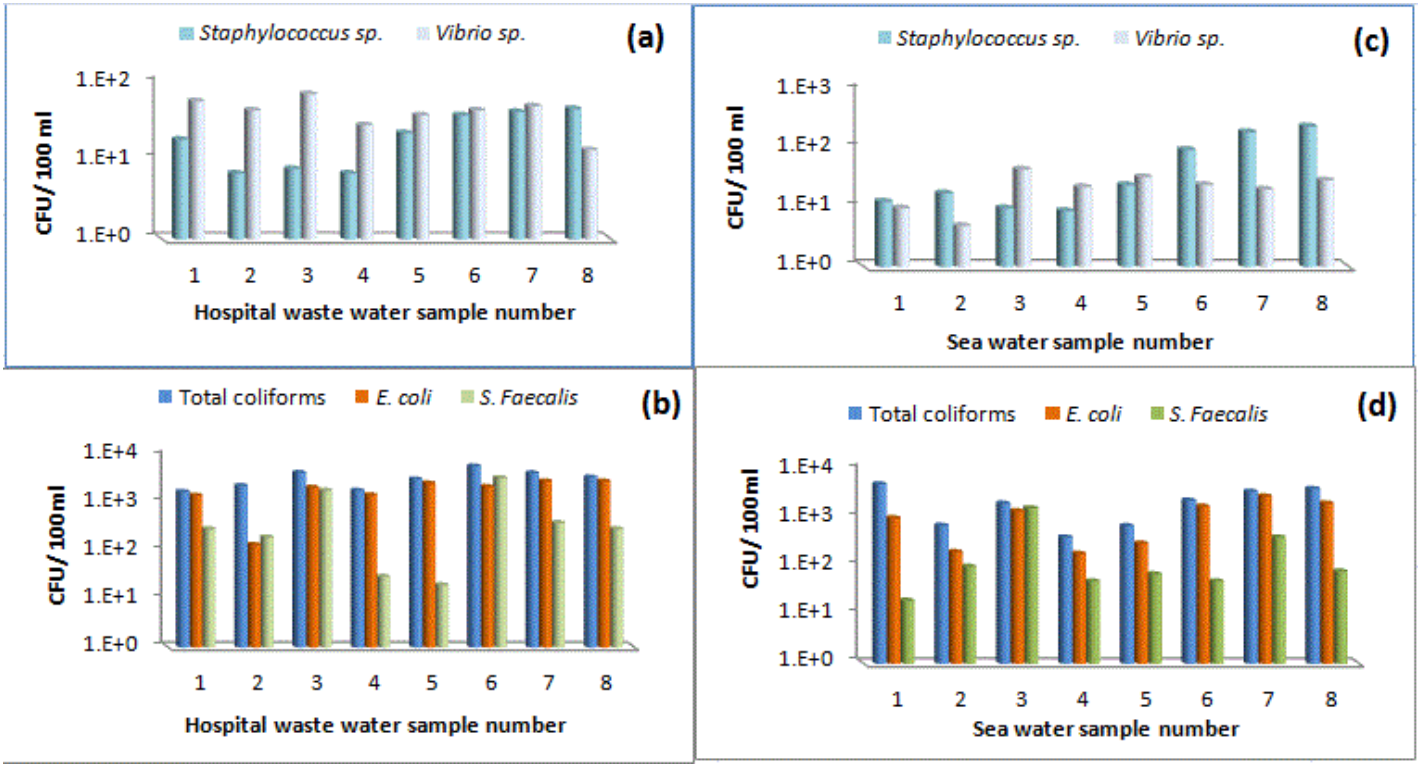

Figure 4. Viable count of pathogens (a) and fecal coliforms (b) in sea water samples, and also pathogens (c) coliforms (d) in hospital waste water samples. Values are average (n=3).

fecal indicators. The counts of total coliforms (TC), *Escherichia coli* (EC) and fecal *Streptococci* (FS) fluctuated between $1.8x10^3$ and $6.2x10^3$ CFU/100 ml for TC, $1.4x10^2$ to $3.0x10^3$ CFU/100 ml for EC and $2.0 x10^1$ to $3.5x10^3$ CFU/100 ml for (FS), (Figure 4b).

The general bacteriological picture in sea water samples showed that the density of *Staphylococcus* sp. ranged from $0.9x10^1$ to $2.5x10^2$ CFU/100 ml and *Vibrio* sp. was recovered in fewer counts (from $0.5x10^1$ to $4.6x10^1$ CFU/100 ml), (Figure 4c).

Total coliforms, *Escherichia coli* and fecal *Streptococci* were detected in 100% of sea water samples with high densities. The counts fluctuated between $4.0x10^2$ to $5.0x10^3$ CFU/100 ml for TC, between $1.9x10^2$ to $2.9x10^3$ CFU/100 ml for EC, and from $2.0x10^1$ to $1.6x10^3$ CFU/100 ml for FS, (Figure 4d).

The composition of bacteria resistance to different chemicals (heavy metals, antibiotics, dyes and surfactant) was examined in natural bacterial communities from hospital waste water and sea water. The occurrence percentages of pathogens with respect to the total heterotrophic counts in hospital waste water ranged from 0.02 to 0.33%, while coliforms represented in higher percentages ranged from 1.95 to 15.0%. In seawater samples the occurrence percentages of pathogens ranged from 0.04 to 0.12% and coliforms ranged from 0.95 to 29.10%.

The average count of total viable heterotrophic bacteria in hospital waste water was 1.4×10^5 and in sea water was 1.5×10^5 CFU/100 ml. The occurrence percentages of total pathogens and fecal coliforms in hospital waste water (0.11 and 7.8%, respectively) were higher than that in sea water samples (0.08 and 6.09%), (Table 7).

DISCUSSION

Most bacteria have multiple routes to resistance to any drug and, once resistant, can rapidly give rise to vast numbers of resistant progeny (Livermore, 2003). Antibiotic and metal resistance is frequently associated with each other and often strongly correlated. Antibiotic resistant and metal tolerant organisms seem to be present due to the exposure to environment contaminated with metals that causes co-incidental selection for antibiotics and heavy metals resistance factors (Ansari et al., 2014).

Concern for environmental quality has stimulated scientific studies on the biological effects of metal contamination on the marine environment. From the standpoint of environmental pollution, heavy metals and metalloids are extremely toxic because of their relative accessibility to biological systems. Yet, in response to toxic concentrations of heavy metals, many aquatic organisms,

Table 7. The occurrence percentages (%) of pathogens and coliforms with respect to total heterotrophic counts in hospital waste water and sea water samples.

Sample	Waste water			Sea water		
Number	**THVC**	**Pathogen**	**Fecal coliforms**	**THVC**	**Pathogen**	**Fecal coliforms**
1	2.4×10^4	0.33	15.0	2.0×10^4	0.12	29.10
2	2.8×10^4	0.19	9.78	2.1×10^4	0.11	4.81
3	8.0×10^4	0.10	10.78	7.1×10^4	0.08	7.03
4	4.5×10^4	0.08	7.82	$6.7 \times .10^4$	0.05	0.95
5	5.8×10^4	0.11	10.35	5.9×10^4	0.10	1.79
6	2.8×10^5	0.03	4.26	3.1×10^5	0.04	1.33
7	3.2×10^5	0.03	2.45	3.3×10^5	0.07	2.03
8	3.4×10^5	0.02	1.95	3.6×10^5	0.08	1.69
Average	1.4×10^5	0.11	7.80	1.5×10^5	0.08	6.09

THVC, Average count of total viable heterotrophic bacteria (CFU/100 ml).

including microorganisms, can develop resistance and the ability to degrade or utilize these substances (Sabry et al., 1997; Hookoom and Puchooa, 2013).

It can be assessed that most bacterial isolates were sensitive to Hg but they were highly resistant to Cr, Ba, Ni and Cu. Although some of them act as essential micro nutrients for living beings, at higher concentrations they can lead to severe poisoning (Hookoom and Puchooa, 2013).

Multiple metal resistances also seemed to be the rule rather than the exception. All bacterial isolates were resistant to more than one metal ions but penta-metal-resistant bacteria were exhibited only in hospital waste water isolates. Similar observations were previously reported (Sabry et al., 1997; De Souza et al., 2006; Kacar and Kocyigit, 2013).

Emergence of bacteria resistant to antibiotics is common in areas where antibiotics are used, but occurrence of antibiotic resistance bacteria is also increasing in marine environment. This is why many studies have been recently carried out to determine the distribution of antibiotic resistance bacteria in freshwater basins, estuaries, municipal drinking water and sewage waters. However, there is relatively very few studies on antibiotic resistant bacteria in marine water (Manivasagan et al., 2011).

Antibiotic resistance of heterotrophic bacteria isolated from the surface and subsurface water of estuarine was determined. The levels of resistance of bacteria to various antibiotics differed considerably (Mudryk, 2002).

The current study has displayed that bacteria inhabiting the hospital waste water were much more resistant to antibiotics than those isolated from polluted sea water. In hospital waste water, the response of the heterotrophic bacteria to five tested antibiotics ranged from complete resistance to total sensitivity, where, the bacterial resistance was noted in the cases of ceftriaxone, levofloxacin vancomycin, and amikacin, while at the same time the bacteria were most sensitive to imipenem while in sea water a high level of bacterial resistance to vancomycin was noted with a simultaneous high sensitivity to ceftriaxone, levofloxacin, amikacin and imipenem.

Similarly, the bacterial resistance was reported by Sabry et al. (1997) which showed that the response of the sea water isolates to 11 tested antibiotics ranged from complete resistance to total sensitivity and multiple antibiotic resistance was exhibited by 70.4% of the total isolated population The highest incidence of metal-antibiotic double resistance existed between lead and all antibiotics (100%), copper and penicillin (95%) and nickel and ampicillin (83.3%).

Microorganisms resistant to both metals and antibiotics have been isolated frequently from different environments and clinical samples (Sabry et al., 1997; Kacar and Kocyigit, 2013). As a result new phenotypes are inherited in communities which develop under the influence of pollutants. The presence of metal and/or antibiotic-resistant bacteria in natural habitats can pose a public health risk (Sabry et al., 1997).

Most antibiotic resistant microbes emerge as a result of genetic change and subsequent selection processes by antibiotics. The resistance factor may be chromosomal, that developed as a result of spontaneous mutations and extrachromosomal resistance (plasmid resistance) (Selim et al., 2013).

Also, to face heavy metals profusion in the environment, bacteria have evolved several resistance mechanisms that lead to persist or/and to grow are in several cases plasmid-borne. These plasmid mediated resistance to heavy metals can also carry genes coding for antimicrobial resistance (Karbasizaed et al., 2003; Habi and Daba, 2009).

The present results have proven that, the incidence of multiple resistances either to metal or antibiotics was observed in heterotrophic bacteria inhabiting hospital waste water, while heterotrophic bacteria inhabiting sea water were characterized by single antibiotic resistance and multiple metal resistances. In hospital waste water, most of the bacteria were resistant to 2-3 antibiotics and

3-5 metals (at 1 ppm). Moreover, in seawater samples, most of the bacteria were resistant to only one antibiotic and 3-4 metals (at 1 ppm).

One environmental concern related with hospital effluents is their discharge without preliminary treatment. The presence of antibiotics may enhance the effectiveness of multi-drugs resisting bacteria (Almeida et al., 2014). Multidrug-resistant strains are critical to the total accumulation of resistance (*Streptococcus pneumoniae*, methicillin-resistant *Staphylococcus aureus*) (Livermore, 2003).

Bacteria inhabiting many water basins are characterized by multiple antibiotic and metal resistance (Mudryk, 2002; Ansari et al., 2014). Multiple resistance is a phenomenon whose mechanisms have not yet been well recognised. Multiple resistances may be coded on plasmids, mutational events or on even smaller and mobile genetic elements called transposons. Transposons are able to move between plasmids and bacterial chromosomes. The bacterial strains inhabiting the waters of lake are characterised by multiple antibiotic resistance. This indicates that estuarine bacteria are perfectly capable of detoxicating these antibacterial substances (Mudryk, 2002).

Most of heterotrophic bacterial isolates have high incidence of metal-antibiotic double resistance, also these isolates exhibited multiple resistance to different dyes as crystal violet, iodine and sufranine and other chemicals as SLS surfactant. In the same trend, De Souza et al. (2006) reported that, mercury resistant bacteria are also resistant to many antibiotics and other toxic chemicals. In addition, it has long been reported that bacteria inhabits in industrial effluents utilizing its constituents as their source of energy (Hassan et al., 2013). The microorganisms used in most of these studies were *Staphylococcus sp., E. coli, Bacillus sp., Clostridium* sp., and *Pseudomonas* sp. in bacteria (Marimuthu et al., 2013).

Adams (1967) confirmed that crystal violet has an antibacterial action against *E. coli, S. aureus, S. faecalis* and *B. subtilis*. The effect of the dye was measured as minimum inhibitory concentration or retardation of growth. On the other side, Lachapelle et al. (2013) concluded that antiseptics have broader spectrums of antimicrobial activity than antibiotics.

Povidone iodine has the broadest spectrum of antimicrobial activity. Corwin et al. (1971) stated that the sensitivity to SLS of *Shigella flexneri* and *E. coli* is determined by at least three genes. A major difference between the two strains is their relative resistance to the anionic detergent, sodium lauryl sulfate.

The composition of bacteria resistance to different chemicals (heavy metals, antibiotics, dyes and surfactant) was examined in natural bacterial communities from hospital waste water and sea water. Obviously, the occurrence percentages of total patho-gens and fecal coliforms in hospital waste water (0.11 and 6.31%, respectively) were higher than that in sea water samples (0.08 and 4.64%).

Species composition of water samples could affect antibiotic resistance pattern. Significant differences to some antibiotics of *Enterobacteriaceae* strains isolated from different sites were not related to fecal pollution level. Also, global resistance to metals was not influenced by fecal pollution (Habi and Daba, 2009). In recent decades, the antimicrobial resistance of bacteria isolated from hospital increased. Gram negative bacterial strains are the most frequent bacterial strains (Selim et al., 2013).

Conclusion

Depending on the metal and antibiotic resistance of heterotrophic bacteria inhabiting hospital waste water and polluted seawater, the current study observed high incidence antibiotics-metals double resistance among heterotrophic bacteria inhabiting hospital waste water comparing with polluted seawater. Also these isolates exhibited multiple resistances to different dyes as crystal violet, iodine and sufranine and other chemicals as SLS surfactant.

As a result new phenotypes are inherited in communities which develop under the influence of pollutants. One environmental concern related with hospital effluents is their discharge without preliminary treatment. The finding suggests that hospital waste water is an important source for the spread of new phenotype bacteria with mutiple-resistance. The presence of new phenotype bacteria with mutiple-resistance in natural habitats can pose a public health risk.

Conflict of interest

The author(s) have not declared any conflict of interests.

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Vol. 8(42), pp. 3625-3629, 15 October, 2014
DOI: 10.5897/AJMR2014.7106
Article Number: 2B9170748175
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Detection of cockroaches as mechanical carrier of *Escherichia coli* and *Salmonella* species

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Received 4 September, 2014; Accepted 13 October, 2014

Cockroaches are cosmopolitan and are found in warm snug places. They are active at night and like to live in humid cracks, behind appliances, cabinets and drawers. They carry microbes on their external body surfaces as they often live in unsanitary places and are thus dangerous to human. This study was carried out in Quetta city. 50 samples of cockroaches were collected from washroom, kitchen and gardens from different areas of Quetta city. The collected cockroaches were brought to the laboratory for further investigation. The cockroaches were inoculated in brain heart infusion (BHI) broth and were incubated at 37°C for 24 h. The subcultures were made on Mac Conkey, SS and EMB agar for the isolation and purification of microorganisms. Biochemical tests that were catalase, simon citrate, methyl red and Voges-Proskauer (MR-VP) and motility tests were applied and the results show the presence of 82% *Salmonella* and 64% *Escherichia coli* on the external surfaces of cockroaches. It is deduced from the study that cockroaches are vital carriers of infectious pathogens; therefore, their control is necessary to minimize the spread of such infectious diseases.

Key words: Cockroaches, broth, agar, biochemical tests, *Salmonella*, *Escherichia coli*, pathogens.

INTRODUCTION

Insects are cosmopolitan and have the most successful history of life and exists since Pennsylvanian period (about 325 million years before) (Atkinson et al., 1992, Salehzadeh, 1992; Cloarec et al., 1992; Kopanic et al., 1994; Mohammadi, 1998; Daly et al., 1998; Cochran, 2001). In the world almost 3500 species of cockroaches have become adopted to live in human habitations (Stankus et al., 1990; Ebeling, 1978). Cockroaches consume cheese, meat, starch, grease, vegetables and fruits and are thus omnivores (Chamavit et al., 2011). The habitat, morphology, and mobility of cockroaches make them mechanical vectors for the tansmission of diseases (Fotedar et al., 1991). They transmit diseases through mechanical routes (Rivault et al., 1993). Cockroaches harbor numerous pathogenic and potentially pathogenic bacteria; they may carry bacteria on their cuticle or in the gut (Cloarec et al., 1992). The bacterial load on their bodies may be up to a million and in each of their fecal droppings can be 7 million (Bennett, 1993). Cockroaches are found to be potential vector in spreading nosocomial infections in hospital (Fotedar et al., 1991). Cockroaches are present everywhere, but

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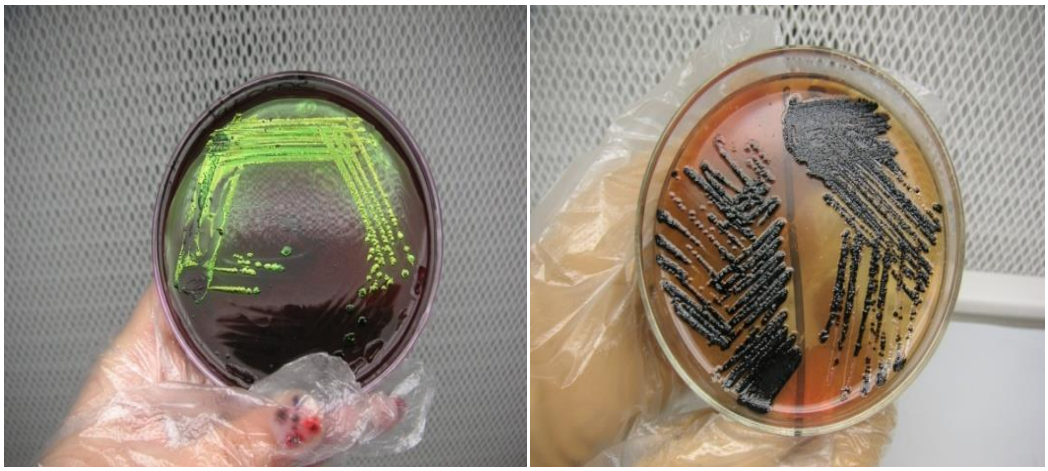


***E.coli* on EMB agar** ***Salmonella* on SS agar**

Figure 1. *E. coli* and *Salmonella* on EMB and SS agar.

mostly in hospitals, homes, kitchens, hotels and restaurants (Burgess, 1984). The increased infestation with the German cockroach (*Blattella germanica*) has caused concern because of its ability of contaminating food with disease causing germs. Thus food hygiene is compromised which results in health hazards (Tanaka and Motoki, 1993). The American cockroach (*Periplaneta americana*) is also one of the possible vectors of bacteria and the sewers are not only safe habitat but also provide them with access to bathrooms and basements (Brenner et al., 1978).

Cockroaches cause many health problems, but two of them are potentially serious. First they cause allergic reaction and second, they are a vector of multidrug resistant pathogens (Tungtrongchitr et al., 2004). For example, members of the genus *Salmonella* (fam. Enterobacteriaceae) can be found in the intestine of the host (Popoff et al., 2004). They are capable to resist dehydration and freezing and can survive for several years in harsh environment (Tortora et al., 2005). *Salmonella* species can persist at least 4 four years in the feces of cockroaches (Rueger and Olson, 1969). Contaminated meat is the most common and most frequent source of infections, caused by *Salmonella* species (Gatto et al., 2006). *Escherichia coli* also belong to the family Enterobacteriaceae and is a gram negative bacterium (the cells get pink color after Gram staining). *E. coli* can remain alive in food and water for a long period. The cells grow best at 37 ^{0}C which is the body temperature of a healthy human. Once they enter the human body they begin to reproduce rapidly, their treatment is restricted (Brown, 1993; Libkin, 1995; Meeker- Lowry, 1995). There are different strains of *E. coli*. The harmless strains are part of our normal flora of gut (Kubitschek, 1990). The harmful strains of *E. coli* cause food poisoning in humans (Vogt and Dippold, 2005). Cockroaches are capable of carrying microorganisms if they come in contact with contaminated materials (Burgess et al. 1973).

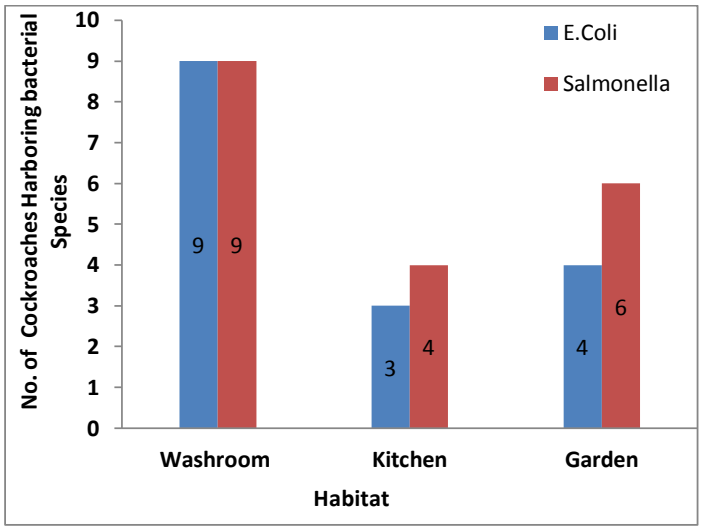
The open conduit carrying sewages are high in Quetta city which is an ideal habitat for cockroaches so the population of cockroaches' carrying infectious pathogen is increasing. This is the first study of its kind conducted in Quetta city to find out the role of cockroaches as mechanical carrier of *Salmonella* species and *E. coli*. Findings of this study would help in assessing the role of cockroaches in spread of enteric disease such as typhoid and food poisioning.

MATERIALS AND METHODS

The study was carried out in Quetta city. Out of 50 cockroaches, 19 (38%) were collected from washrooms, 18 (36%) were collected from kitchens, while only 13 (26%) cockroaches were collected from the garden. Twenty six individuals were American cockroaches and 24 were German cockroaches. The cockroaches were collected by hand randomly from different localities such as washrooms, kitchens and gardens by using rubber gloves. They were kept in sterilized sample bottles then placed those sample bottles until they died to avoid the use of insecticides. The collected cockroaches were brought to the laboratory. They were identified at species level. Cockroaches were kept in brain heart infusion broth (BHI Oxoid UK) for few minutes in order to transfer bacteria from the external surface of cockroaches to the BHI broth (Oxoid UK). The test tubes were incubated at 37°C for 24 h. Subcultures were made from the BHI bottles on Mac Conkey agar (Oxoid UK), Salmonella Shigella agar (SS agar Oxoid UK) and Eosin Methylene Blue agar (EMB Oxoid UK). Prolonged incubation period was given for the maximum chance of isolation of low number of possible bacteria. Morphological characters and biochemical tests identified the grown bacteria (Edwin et al, 1985). *E. coli* has metallic sheen colonies on EMB agar which is selective medium only for *E.coli* while *Salmonella* forms black colonies on SS agar (selective medium for *Salmonella*) (Figure 1). All the isolates, obtained from selective media (EMB and SS), were identified on the basis of their biochemical properties using catalase, Simon citrate, MR-VP and Sulphur Indole Motility test and gram staining reactions test (Talaro, 2007).

Table 1. Total number of American and German cockroaches in different locations

Location	Total number of cockroaches	
	American cockroach	German cockroach
Washroom	10	9
Kitchen	8	10
Garden	8	5
Total	**50**	



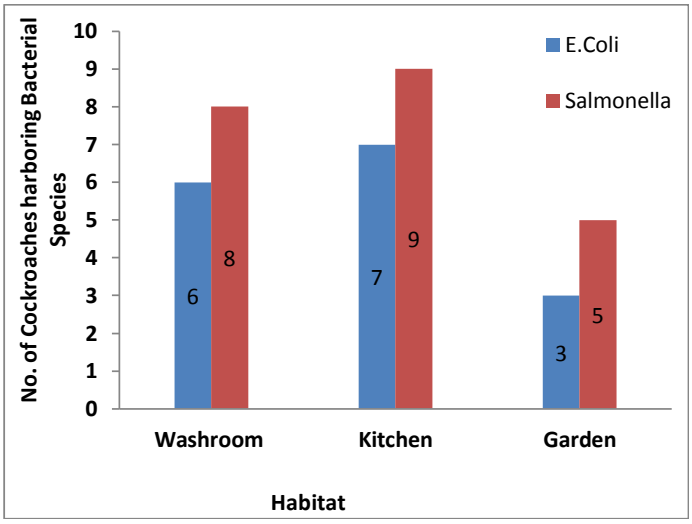
Graph 1. Bacterial species in three habitats isolated from American cockroaches.

RESULTS

Bacterial species were isolated from all 26 American cockroaches, and 24 German cockroaches which were collected randomly from three habitats of several areas (Table 1, Graph 1 and 2). The bacterial species isolated from no. of American and German cockroaches collected from each habitat is represented in Graph 1 and 2 respectively. Among the American cockroaches, maximum number of infested cockroaches was collected from washrooms while minimum no. of infested cockroaches was captured from kitchen. In the case of German cockroaches the maximum no. of cockroaches captured from kitchen have bacterial load while few Garden cockroaches were infested. From the total of 50 collected cockroaches, 32 (64%) showed bacterial load of *E. coli* and, 41 (82%) showed a bacterial load of *Salmonella.* The presence of *E. coli* and *Salmonella* was confirmed by having metallic sheen colonies on EMB agar and black colonies on SS agar respectively. Further biochemical tests were applied using catalase, Simon citrate, MR-VP and Sulphur Indole Motility test to confirm the bacterial species. This study thus revealed that cockroaches contain more bacterial load of *Salmonella* compared to *E. coli* (Graphs 1 and 2).

DISCUSSION

The sampled habitats represent the urban sites where cockroaches come into contact with humans and they also reflect that a cockroach infestation and invasion might play an important role in transmission of bacterial species. Elgderi et al. (2006) isolated 55 species of bacteria from German cockroaches collected from five habitats, and 18 bacterial species were known to be pathogenic or potentially pathogenic. Our study, which was performed in Quetta city, reveals that those localities where cockroaches are found abundantly and come into contact with humans in daily routine play an important role by carrying and spreading bacterial species. The presence of bacterial species on cockroaches in residential habitats might be a temporary reservoir that could lead to a rapid recovery of bacterial population infestation in near future (Hossein et al., 2003). Many studies have revealed the dominance (up to 88%) of isolated Gram-negative bacteria on the surface or cuticle of cockroaches (Paul et al., 1992). Most of the bacteria belong to the family



Graph 2. Bacterial species in three habitats isolated from German cockroaches.

Enterobacteriaceae (Sramova et al., 1992; Rivault et al., 1993).

Our study supports that cockroaches are a mechanical carrier of bacterial species. *E. coli and Salmonella species* were present on the exoskeleton of cockroaches and both belong to family Enterobacteriaceae. Other pathogenic bacteria were also isolated from coakroaches thatis *Klebsiellla* species, *Proteus* species, *Pseudomonas aeruginosa* and *Staphlococcus aureus* (Fotedar et al., 1991). Insecticides and bactericides in more sensitive areas could not inhibit the bacterial species, which are present on cockroaches successfully in the end or even though the cockroaches were killed a lot temporarily there but the quantity of bacteria carried by cockroaches were inhibited because of the decline of cockroach population (Graczyk et al., 2005). Our work suggests that bactericide and insecticide should be used simultaneously and constantly to reduce the cockroach population and bacterial densities in sensitive areas and other disinfectants should also be used at the household level.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Vol. 8(42), pp. 3656-3661, 15 October, 2014
DOI: 10.5897/AJMR2014.7031
Article Number: 19B0A4548185
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Microbiological assessment of bottled water brands in the Bulawayo market, Zimbabwe

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Received 23 July, 2014; Accepted 29 September, 2014

This study aimed at evaluating the microbial quality of bottled water available in the Bulawayo market necessitated by the influx of local and imported bottled water brands into the market. The samples were analysed for *Escherichia coli*, heterotrophic plate count (HPC), total coliforms, feacal coliforms and for *Pseudomonas aeruginosa*. Total coliforms had a prevalence of 10% in local bottled water brands with bacterial counts not exceeding 30 cfu/250 ml. *E. coli* and feacal coliforms had prevalence of 2.5 and 5% respectively with maximum counts of 2 cfu/ml for *E. coli* and 5 cfu/250 ml for feacal coliform counts. *P. aeruginosa* was detected in 8.6% of local brand samples with counts not exceeding 10 cfu/250 ml. For heterotrophic plate count, 78% of the local brand samples had HPC ≤ 100 cfu/ml with counts as high as 2 x 10^3 cfu/ml recorded for the non-conforming samples. In the imported brands, 92% of the samples conformed to HPC ≤ 100 cfu/ml while counts for non-conforming samples ranged between 1.17 x 10^2 and 3.4 x 10^2 cfu/ml. The imported bottled water brands had significantly ($p < 0.05$) lower *P. aeruginosa* and total coliforms counts as compared to local bottled water brands, while there was no significant difference in *E. coli*, heterotrophic plate count and feacal coliform counts. However, based on International Bottled Water Association and WHO drinking water guidelines, *P. aeruginosa*, coliforms and *E. coli* counts in some local and imported bottled water samples exceeded the recommended maximum of no detection. This may have health implications to consumers. The quality of bottled water in the market maybe unsatisfactory for human consumption and warrants the need for strict monitoring and control in the production and trade in bottled water.

Key words: Bottled water, microbial quality, bacteriology, Bulawayo market, Zimbabwe.

INTRODUCTION

Bottled water is sold to consumers in sealed containers. It is obtained from various sources and may be treated to improve on its quality and make it satisfactory for human consumption after which, it is sealed in containers for sale (Abd El-Salam et al., 2008). Such water sources include springs, municipal systems and other sources which may be considered to be of safe and sanitary quality and fit for human consumption (Alfadul and Khan, 2011; Baba et al., 2008).

There is a worldwide upsurge in the use of bottled

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water, a trend also common in Bulawayo, Zimbabwe. It is popular public perception that bottled water is safe for consumption because it is free from contaminants and impurities (Venieri et al., 2006; Zamberlan da Silva et al., 2008; Okagbue et al., 2002). This correlates with the increasing use of bottled water associated with an influx of both local and imported bottled water brands in the Bulawayo market.

The increase in the use of bottled water in Bulawayo is influenced by a number of factors which include water rationing by the local municipality (Bulawayo city council). This is also linked to the fact that, upon reconnection after water rationing, the municipal water comes with a muddy appearance. This is undesirable to the consumers and has resulted in shunning the use of municipal water for drinking purposes. A similar trend, which influences the increase in the use of bottled water has also been noted in Harare, another city in Zimbabwe (Ivanova et al., 2013; WHO, 2006). There are critical water shortages in Harare which have resulted in typhoid and cholera outbreaks since the water is also unsafe for drinking. For consumer protection, there is need to investigate the quality and safety of bottled water and to effect appropriate and adequate monitoring and control mechanisms (Zarocostas, 2008).

During the era of heightened economic challenges Zimbabwe faced in 2008, a lot of companies and industries shutdown only to re-emerge after the dollarisation of the Zimbabwean economy. Local bottle water production was equally affected. Most of the resurgent processing companies are struggling to operate to their full capacity and questions are raised on the quality of products produced since in 2011, a total of 40 bottled water producing companies were banned. Prior to the economic challenges in Zimbabwe, a study conducted in Bulawayo found that total viable counts and coliforms in some locally processed bottled water exceeded their recommended maximum with the detection of a low incidence of *S. aureus, Pseudomonas* spp. and *Bacillus* spp. Despite the study concluding the water to be generally safe, it is evident that there were public health implications (Okagbue et al., 2002).

There is therefore, a need to investigate the physico-chemical and microbiological safety of bottled water in the market to evaluate its health implications. It is paramount for consumers to note that bottled water is not necessarily safer than tap water since they may both originate from the same source and be contaminated with the same microorganisms and other contaminants.

This study sought to determine the bacteriological profile of local and imported bottled water brands in the Bulawayo market to assess on its microbiological safety to public consumption.

MATERIALS AND METHODS

This study was conducted in 2012 in the city of Bulawayo, Zimbabwe to assess the microbial quality of popular bottled water brands. The samples were focused on local bottled water brands coded; local A, B, C and D and on imported bottled water brands coded; import E, F, G, H and I.

Analysis of bottled water was done thrice per month over a period of eight months. Corresponding to ten city supermarkets and six retail outlets involved in the study, three samples of unexpired bottled water were purchased for each brand available and transported to the laboratory for microbiological analysis in a cooler box. The samples were analysed for *Escherichia coli*, heterotrophic plate count, total coliforms, faecal coliforms and *Pseudomonas aeruginosa*. Consequently, one hundred and forty four samples were analysed per brand per month.

Bacteriological analysis

The bottled water samples were analysed through the membrane filtration technique for total coliforms, faecal coliforms and *P. aeruginosa*. The pour plate technique was used for the analysis of *E. coli* and heterotrophic plate count.

In the determination of *E. coli,* the EMB agar (BIOLAB) was used. Plating involved the inoculation of 1 ml aliquots from serially diluted bottled water samples into EMB plates and incubating for 24 h at 37°C. The IMViC tests were carried out as confirmatory tests for the characteristic growing colonies and the counts expressed in cfu/ml. The heterotrophic plate counts were determined through the pour plate technique in which 1 ml aliquots from serially diluted bottled water samples were mixed with 10 ml of molten standard methods agar (plate count agar) and poured into Petri dishes. The plates were incubated at 37°C for 48 h. The pour plate counts were expressed in cfu/ml.

To determine the total coliforms, membrane filters were used to filter 250 ml of bottled water corresponding to each water sample after which they were plated on m-endo agar (Difco) at 37°C for 24 h. For the determination of faecal coliforms, the membrane filters were plated on m-FC agar (Difco) at 44.5°C for 24 h. For *P. aeruginosa* the membrane filters were plated on Pseudomonas CN selective agar supplemented with cetrimide and nalixidic acid (Merck) and incubated at 37°C for 24 h. The growing colonies were confirmed by the oxidase test, acetamide broth and King's B medium (Merck). The membrane filter counts were expressed in cfu/250 ml.

The difference between bacterial counts in local and imported bottled water brands was analysed through the independent samples t-test corresponding to each bacterial parameter investigated.

RESULTS AND DISCUSSION

In local bottled water brands, there was 10% mean prevalence for total coliforms with the detections distributed (Table 1) throughout the four local brands while *E. coli* and the feacal coliforms had a mean prevalence of 2.5 and 5%, respectively. Local A bottled water brand exhibited the highest prevalence in all the bacterial parameters (Figure 1). The results from this study are consistent to findings of bottled water microbial quality in Egypt (Abd El-Salam et al., 2008).

Bottled water standards such as the International Bottled Water Association (IBWA) require bottled water which is sold as a foodstuff, to be free from parasites and pathogenic bacteria which render it unfit for human consumption. These bacteria include *E. coli*, *P. aeruginosa* and other coliforms and faecal streptococci in

Table 1. Mean monthly bacterial counts in local and imported bottled water brands.

Bottled water brands	Bacterial counts (monthly mean)	Month							
		1	2	3	4	5	6	7	8
Local A	Total coliforms (cfu/250 ml)	5	0	0	3	0	1	0	0
	E. coli (cfu/ml)	1	0	0	0	0	0	0	0
	Feacal coliforms (cfu/250 ml)	1	0	0	1	0	0	0	0
	P. aeruginosa (cfu/250 ml)	0	0	0	9	0	0	2	0
	Heterotrophic plate count (cfu/ml)	1.3×10^2	68	74	2×10^2	84	59	81	9.6×10^2
Local B	Total coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	E. coli (cfu/ml)	0	2	0	0	0	0	0	0
	Feacal coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	P. aeruginosa (cfu/250 ml)	0	0	0	0	0	0	0	0
	Heterotrophic plate count (cfu/ml)	25	40	36	54	1.1×10^2	23	43	35
Local C	Total coliforms (cfu/250 ml)	0	4	1	0	0	0	0	0
	E. coli (cfu/ml)	0	0	0	0	0	0	0	0
	Feacal coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	P. aeruginosa (cfu/250 ml)	0	0	0	0	0	0	0	0
	Heterotrophic plate count (cfu/ml)	76	85	63	88	92	87	74	49
Local D	Total coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	E. coli (cfu/ml)	0	0	0	0	0	0	0	0
	Feacal coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	P. aeruginosa (cfu/250 ml)	0	7	0	0	0	1	0	0
	Heterotrophic plate count (cfu/ml)	50	47	75	34	86	38	40	52
Import E	Total coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	E. coli (cfu/ml)	0	0	0	0	0	0	0	0
	Feacal coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	P. aeruginosa (cfu/250 ml)	0	0	0	0	0	0	0	0
	Heterotrophic plate count (cfu/ml)	68	54	27	78	39	82	76	43
Import F	Total coliforms (cfu/250 ml)	0	0	0	2	0	0	0	0
	E. coli (cfu/ml)	0	0	0	0	0	0	0	0
	Feacal coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	P. aeruginosa (cfu/250 ml)	0	0	0	0	0	0	0	0
	Heterotrophic plate count (cfu/ml)	44	19	56	20	68	34	60	37
Import G	Total coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	E. coli (cfu/ml)	0	0	0	0	0	0	0	0
	Feacal coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	P. aeruginosa (cfu/250 ml)	0	0	0	0	0	0	0	0
	Heterotrophic plate count (cfu/ml)	95	82	43	99	64	51	89	17
Import H	Total coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	E. coli (cfu/ml)	0	0	0	0	0	0	0	0
	Feacal coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	P. aeruginosa (cfu/250 ml)	0	0	0	0	0	0	0	0
	Heterotrophic plate count (cfu/ml)	52	66	71	37	80	28	94	45
Import I	Total coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	E. coli (cfu/ml)	0	0	0	0	0	0	0	0
	Feacal coliforms (cfu/250 ml)	0	0	0	0	0	0	0	0
	P. aeruginosa (cfu/250 ml)	0	0	0	0	0	0	0	0
	Heterotrophic plate count (cfu/ml)	81	50	25	63	73	61	39	56

a sample of 250 ml; *Clostridium* species in a sample of 50 ml (Makris et al., 2013; Reyes et al., 2008). WHO standards require total coliforms and feacal coliforms to be undetectable per 100 ml of bottled water while total

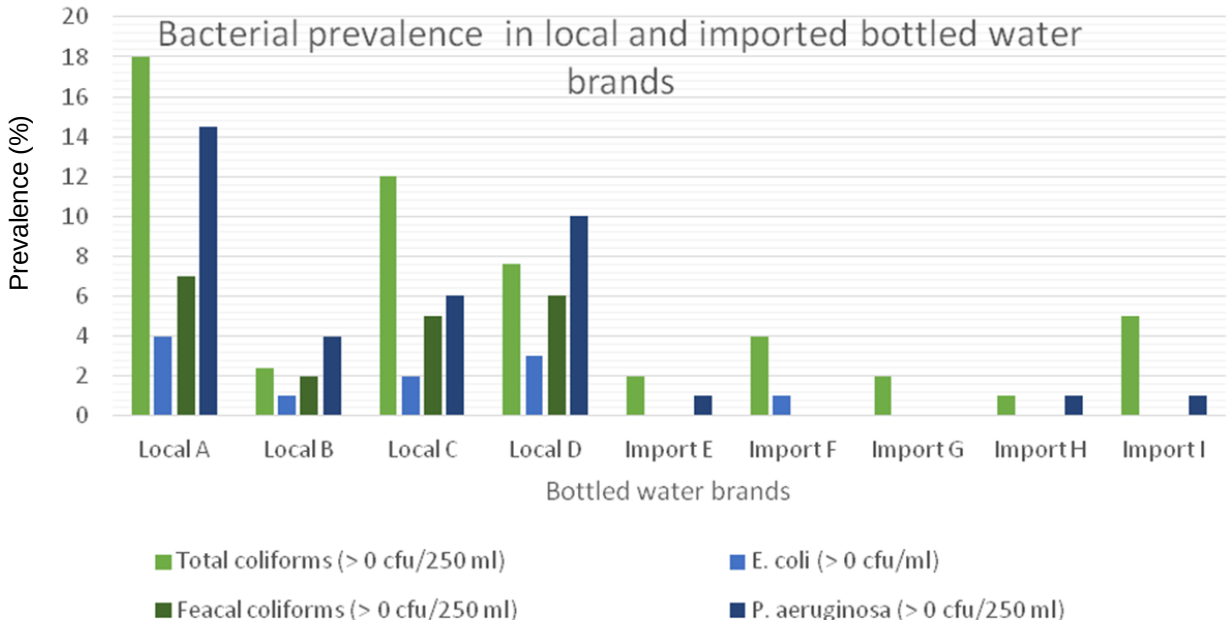

Figure 1. Bacterial prevalence in local and imported bottled water brands in relation to bacterial detection limit.

viable counts (TVC) of organisms in bottled water should not exceed 100 cfu per ml (WHO, 2006). Considering that some bacterial parameters which were tested exceeded the WHO detection limits, this may have health implications to the consumers. This is particularly true for local brands (Figure 1) which had most samples not complying with WHO guidelines as compared to the imported bottled water brands.

As compared to local bottled water brands, lower prevalence was recorded in imported bottled water for total coliforms having a prevalence of 2.8%, *E. coli* (0.2 %) while there were no detections for the feacal coliforms. Statistically, imported bottled water brands had significantly ($p < 0.05$) lower *P. aeruginosa* and total coliforms counts as compared to local bottled water brands, while there was no significant difference in *E. coli*, heterotrophic plate count and feacal coliform counts. This implies that imported bottled water brands present lesser health implications to the consumers. Consequently, in the Bulawayo market, imported bottled water brands are generally safer than the locally processed bottled water brands.

Higher microbial incidence in local brands of bottled water can be attributed to non-compliance to hygienic practices by local industries a situation supported by the banning of 40 water bottling companies in Zimbabwe in the year 2011. Failure to detect feacal/thermotolerant coliforms and a very low incidence of *E. coli* in imported bottled water samples tested may mean that the samples are free from feacal contamination and that the bottled water may be microbially safe for consumption by the public (Abdulraheem et al., 2012; Ahmad and Bajahlan, 2009; Al-Omran et al., 2013).

The detection of coliforms in bottled water as an indicator has health implications since this may suggest that there is a potential presence of pathogenic enteric microorganisms such as *Vibrio cholera* and *Salmonella* (Abd El-Salam et al., 2008; Baba et al., 2008; Bhaduri and Sharma, 2014; Bharath et al., 2003). Gastro-enteritis epidemics caused by such pathogens in contaminated bottled water have been documented and they are a problem to children, the elderly and immunocompromised or other vulnerable people (Zeenat et al., 2009; Zhao et al., 2004).

In Trinidad, the bottled water which had 5% prevalence for coliforms was considered unfit for human consumption (Bharath et al., 2003). Based on the recommended zero tolerance for coliforms in potable water, the local bottled water brands in the Bulawayo market can be considered unsafe for human consumption corresponding to the following prevalence for total coliforms (Figure 1); Local A (18 %), Local C (12 %) and Local D (7.6 %) as compared to the ones in imported brands; Import E (2 %), Import G (2 %) and import H (1%).

P. aeruginosa and HPC are used for process management and not necessarily as health indicators (Diduch et al., 2013). *P. aeruginosa* is a possible indicator of unsatisfactory source water or bottling and can cause opportunistic infections. HPC bacteria are potential indicators of overall sanitation in bottled water production and are harmless themselves. However there is a health concern that immune-compromised persons may be at risk from excessive exposure to some of these harmless bacteria (Semerjian, 2011; Zamberlan da Silva et al., 2008).

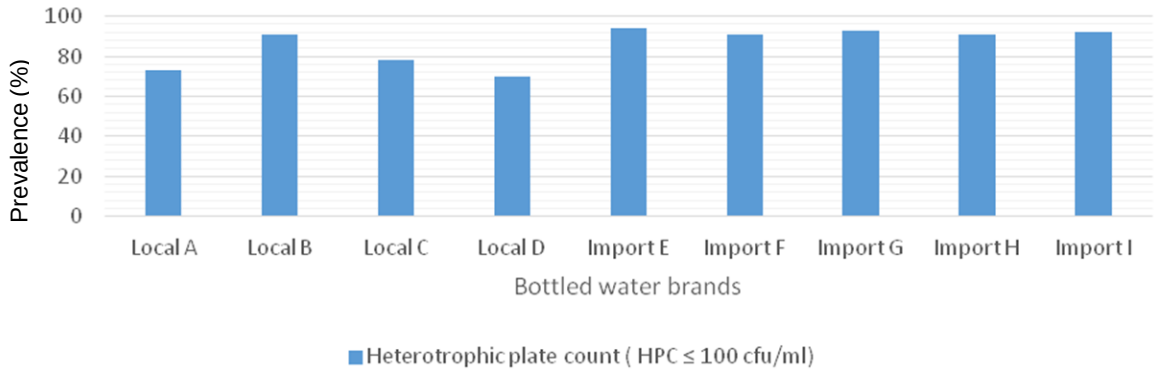

Figure 2. Heterotrophic plate count (HPC ≤ 100 cfu/ml) prevalence in local and imported bottled water brands.

The mean prevalence of *P. aeruginosa* in local bottled water brands was 8.6% while for imported brands it was 0 .6% with the prevalence in different brands illustrated in Figure 1. Since standards require zero prevalence for *P. aeruginosa*, higher prevalence in local brands may suggest that there is non-compliance with good manufacturing practices as reported in other studies (Abd El-Salam et al., 2008; Nunes Filho et al., 2008; Pontara et al., 2011; Raj, 2005). In a study conducted in Bulawayo in 2002, *P. aeruginosa* was found to have 6.7% prevalence (Okagbue et al., 2002).

For HPC, 78% of local bottled water samples had HPC ≤ 100 cfu/ml with the trends for individual brands illustrated in Figure 2. However, of concern amongst the HPC non- conforming samples are those in Local A where some samples had HPC > 2 X 10^3 cfu/ml as compared to other local brands and imported bottled water brands. This is consistent with the prevalence observed for the total coliforms (Figure 1) and implicates the manufacturing process for Local A as responsible for the contamination of bottled water.

As compared to local brands, a greater number of imported brand samples (mean prevalence 92.2%) were within the WHO limit HPC ≤ 100 cfu/ml with individual prevalence of the different brands shown in Figure 2; counts in HPC non-conforming samples, ranged between 1.17 x 10^2 - 2.4 x 10^2 cfu/ml. This is in line with lower prevalence detected for other bacterial parameters analysed for the imported brands despite the fact that there was no significant difference ($p > 0.05$) in HPC counts between local and imported bottled water brands. It can be implied in general that local bottled water processing is not compliant to good manufacturing and hygienic practices and that the sources from which the water is obtained maybe unsatisfactory for human consumption (Rakhmanin and Mikhailova, 2011; Ramalho et al., 2001; Reyes et al., 2008).

The findings for HPC concur with other studies which reported that heterotrophic plate counts of bottled (non-carbonated) water are high and variable (Duranceau et al., 2012; Sakai et al., 2013). A study conducted in Bulawayo reported that, total viable counts where in the range 104 to 151 cfu/ml which is above the bottled water standard of < 100 cfu/ml (Okagbue et al., 2002). In a study conducted in South Africa, HPC were < 100 cfu/ml for most bottled water brands tested, though, one brand of was found to have HPC as high as 2.58 x 10^3 to 1.31 x 10^4 cfu/ml (Ehlers et al., 2004).

To improve the safety of the public to bottled water consumption, there is need for stricter regulation of the bottled water industry and monitoring of imported brands. Enforcement of local industries to comply and be certified for quality and hygiene standards such as the QMS: ISO 9000, FSMS: ISO 22 000 and HACCP should be effected. This would improve the manufacturing process and ultimately the quality of the products produced.

Conflict of interest

The author(s) have not declared any conflict of interests.

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Vol. 8(42), pp. 3650-3655, 15 October, 2014
DOI: 10.5897/AJMR2014.7021
Article Number: D69409848183
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Effects of fertilizers and soil amendments on the incidence of Sclerotinia stem rot in Indian mustard (*Brassica juncea* L.)

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Received 18 July, 2014; Accepted 29 September, 2014

Indian mustard [*Brassica juncea* (L.)] is a major oilseed crop, grown in rabi season in India. Sclerotinia stem rot, stem blight or white rot disease caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is a serious problem in mustard crop in Northern India. The present investigations were undertaken to understand the role of fertilizers and organic amendments on disease development in mustard crop under field conditions. In experiment 1, the effect of nitrogen, phosphorus and potash fertilizers with different levels on the development of Sclerotinia stem rot and on seed yield was studied during two years. The treatment was a combination of 80 kg N, 60 kg P and 60 kg K ha^{-1} which was applied significantly superior over control (without fertilizers) in disease control (60.42%) and increased seed yield (13.80 q ha^{-1}). Application of 100 kg N, and 60 kg P ha^{-1} without K was found to be least effective for disease management. In experiment 2, effect of five soil amendments, that is, mustard cake, neem cake, vermicompost, farm yard manure and sulphur on the development of Sclerotinia stem rot was studied. Neem cake applied at 1 ton ha^{-1} was found significantly superior with minimum disease incidence (20.43%), minimum disease intensity (15.00%), maximum disease control (63.27%) and increased seed yield (14.07 q ha^{-1}) over control (without amendments). The vermicompost and FYM applied at 15 ton ha^{-1} were also found effective in minimizing disease intensity and increased seed yield.

Key words: Fertilizers, Indian mustard, soil amendments and Sclerotinia stem rot.

INTRODUCTION

Indian mustard [*Brassica juncea* (L.) Czern & Coss] is a major oilseed crop grown in rabi season in various regions of India. Rapeseed and mustard are the major oilseed crops of India with oil contents ranging between

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30 and 46%. Among the annual edible oilseeds, rapeseed and mustard contributes about 23% in acreage and over 25% in production for the last five years in India. India holds a leading position in area and production of rapeseed and mustard, that is,5.92 million hectares and 6.78 million tonnes, respectively, with an average productivity of 1145 kg ha-^{1}. The main mustard producing states in India are Rajasthan, Uttar Pradesh, Punjab, Haryana, etc. Among these states, Rajasthan stands first both in area and production, that is, 2.50 million ha and 2.97 million tonnes, respectively, with an average productivity of 1187 kg ha-^{1} (Directorate of Economics and Statistics, 2012). Rapeseed and mustard seeds are commonly used for the extraction of edible oil in Northern India. The oil content of the seeds ranges from 30 to 46%. The seed is used as a condiment in the preparation of pickles and for flavouring curries and vegetables. The oil cake is mostly used as a cattle feed. The seeds, oil and oil cakes are needed not only to meet the demand for internal consumption but also for export in order to earn the much needed foreign exchange.

Sclerotinia stem rot, or stem blight or white rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary has become a serious problem in mustard crop in northern India. *S. sclerotiorum* is soil-borne and has a large host range inciting more than 400 plant species (Boland and Hall, 1994). Sclerotinia stem rot is an economically important yield reducing disease that has been widely reported in the last few years in India and elsewhere. The high disease incidence and severe yield losses discourage farmers to grow the crop (Krishnia et al., 2000). The disease symptoms usually appear four to six weeks after sowing or at flowering stage, when significant damage has already been done. Sudden drooping of leaves followed by drying of plants are characteristic features of the disease. This disease is gaining importance in the mustard growing areas, which may cause crop failure as the disease incidence was recorded up to 73.8% in some districts of Punjab and Haryana (Kang and Chahal, 2000; Sharma et al., 2001). In Rajasthan, the disease incidence was recorded up to 60% (Ghasolia et al., 2004; Shukla, 2005). Various soil factors, such as nutritional status of the soil play a major role in the disease incidence. Sclerotia are the primary survival structures of *S. sclerotiorum* and act as a source of infection for many years in the field. Balanced fertilization and amendments of soil with decomposable organic matter could be an effective method of changing the soil and rhizosphere environment. Composts enrich the soil with microflora potentially competitive or antagonistic to pathogens or release inhibitory substances or volatiles during decomposition. The control of Sclerotinia stem rot of mustard in India is mainly achieved by using fungicides and cultural methods such as crop rotation and tillage to reduce inoculums of the pathogen. Most of the conventional methods are not effective in the management of S. sclerotiorum thus, the present study was undertaken to find out role of fertilizer doses (NPK) and soil amendments (neem cake, mustard cake, vermicompost, farm yard manure and sulphur dust) on disease severity and yield of mustard.

MATERIALS AND METHODS

Inoculum

An isolate of *S. sclerotiorum* was obtained from diseased mustard plant collected from farmer's field. Small pieces of diseased tissues along with adjoining healthy area and sclerotia found in diseased stem were surface sterilized by dipping in mercuric chloride solution (1:1000) for two minutes followed by three washings with sterile water and blot dried then plated aseptically on Potato Dextrose Agar (PDA) in Petri dishes. These were incubated in BOD incubator for growth of the fungus at 27 ± 2°C.

Sub cultures from pure peripheral growth were made on PDA slants. The pathogenicity of the isolated fungus was proved by mixing 15 day old inoculum (grown on sterilized sorghum grains) at the rate of 50 g per pot in the upper 3-5 cm layer of the sterilized soil of each pot. The soil of the pots were moistened and covered with polythene bags and left for 24 h in green house. On next day, apparently healthy surface sterilized mustard seeds were sown in these pots. Re-isolations from the diseased seedlings yielded the same fungus.

Mass multiplication of inoculum

The fungus inoculum was multiplied on sterilized sorghum grains. The sorghum grains were soaked in sterilized water overnight. The excess water drained out. Forty grams of grains was taken in each 250 ml conical flask, plugged with cotton and sterilized in an autoclave at 1.045 kg/cm^{2} pressure for 20 min. The sorghum grains in flasks were inoculated aseptically with 5 days old mycelial discs (5 mm) of the pathogen and incubated for 15 days at 20 ± 2°C. The inoculum was mixed in rows at the time of sowing.

Two experiments were executed during rabi season of 2007-08 and 2008-09 at the research farm of the College of Agriculture, Swami Keshwanand Rajasthan Agricultural University, Bikaner. A randomized block design was used with three replications in 4 × 3 m^{2} plots. The susceptible mustard cultivar 'varuna' was used for all experiments. All experiments were artificially inoculated with 20 g inoculums per meter row. All recommended agronomic practices were followed to raise the crop except fertilizers dose and organic amendments. The fertilizers and organic amendments were applied according to the treatments. Sowing of the crop was done during last week of October.

In experiment 1 the relationship of different fertilizers (NPK) and their combination with the disease development of Sclerotinia stem rot on mustard under field conditions were tested. The different treatments on combination of fertilizers N:P:K (kg ha-^{1}) viz. T_1-60:30:30, T_2-60:30:60, T_3-60:60:30, T_4-60:60:60, T_5-80:30:30, T_6-80:30:60, T_7-80:60:30, T_8-80:60:60, T_9-100:30:30, T_{10}-100:30:60, T_{11}-100:60:30, T_{12}-100:60:60, T_{13}-100:60:0 and T_{14}-80:30:0 were applied with control plot (T_{15}-without fertilizers). Half quantity of nitrogen, full quantity of phosphorus and potash were applied at the sowing time and remaining half quantity of nitrogen were applied in two splits at first and second irrigation.

In experiment 2, the effect of different soil amendments and their doses on disease development of Sclerotinia stem rot was studied. T_1&T_2-mustard cake 1 & 2 tons ha-^{1}, T_3&T_4-neem cake 0.5 & 1 ton ha-^{1}, T_5&T_6-vermicompost 10 & 15 tons ha-^{1}, T_7&T_8-FYM 10 & 15 tons ha-^{1} and T_9&T_{10}-sulphur 30 & 60 kg ha-^{1} were compared against a control plot (T_{11}-without organic amendments). All the

amendments were applied in the plots before sowing of the mustard crop.

The observations of disease intensity, incidence and seed yield were recorded on plot basis. Disease intensity and incidence were recorded after 75 to 90 days after sowing. The results of experiments were statistically analyzed by using appropriate designs. To assess the Sclerotinia rot intensity, the rating (0-4) scale (Lesovoi et al., 1987; Sansford, 1995) was followed. 0 for healthy (No visible lesion), 1 for 0.1-2.0 cm lesion length on the stem, 2 for 2.1 - 4.0 cm lesion length on the stem, 3 for 4.1 - 6.0 cm lesion length on the stem and 4 for more than 6 cm lesion length on the stem or complete griddle plant. The length of lesion on infected stem was considered for recording the disease intensity (Sharma, 1987). The infected area was calculated from five randomly selected plants in each plot and then the average for each treatment was worked out. Percent disease intensity was calculated by using the formula [(sum of individual ratings/no. of plants observed × maximum disease rating) × 100]. The percent disease control was calculated by using the formula [(Disease in control - Disease in treatment/ Disease in Control) × 100] (McKinney, 1923).

RESULTS AND DISCUSSION

Experiment 1 revealed that the application of 80 kg N, 60 kg P and 60 kg K ha-^{1} was significantly superior in percent disease control (p=0.05) as well as in seed yield (13.80 q ha-^{1}) (p=0.05) over control (Table 1). Application of 100 kg N, and 60 kg P ha-^{1} without K was least effective for disease control (4.17%). Application of 80 kg N, 30 kg P without potash resulted in the lowest seed yield (6.59 q ha-^{1}). Table 1 suggests that application of more than 80 kg N ha-1 increase disease severity and reduced the percent disease control. This observation is in accordance with earlier findings of Gupta et al. (2004) that reported that increased nitrogen levels of nitrogen (60 to 90kg ha-^{1}) in soil led to significant increase in Sclerotinia stem rot disease incidence in mustard. In addition, Rathore and Chandawat (2003) found that increased nitrogen fertilization reduces seed yield and stover yield and increase susceptibility of blond psyllum to *Peronospora alta*, causing downy mildew. Chattopadhyay et al. (2002) observed that soil application of K fertilizer in *S. sclerotiorum* infected fields reduced the disease incidence in mustard. Banyal et al. (2008) reported nitrogen fertilization enhanced the development of collar rot caused by Sclerotium rolfsii in tomato, whereas phosphorus and potassium fertilization decreased the disease. Potassium (K) is essential for the synthesis of proteins, starch and cellulose in plants. Cellulose is a primary component of cell walls, and K deficiency causes cell walls to become leaky, resulting in high sugar (starch precursor) and amino acid (protein building blocks) concentrations in the plant. Nitrogen (N) is a key component of amino acids; therefore, an excessive supply of N can bring about higher amounts of amino acids and other N-containing compounds in plant tissues. These mineral imbalances lower resistance to fungal diseases by creating a more favorable environment for pathogens (Span and Schumann, 2010).

Experiment 2 revealed that the addition of neem cake, vermicompost, FYM, organic sulphur and mustard cake significantly decreased the disease intensity (p=0.05) in mustard as compared to the control. The addition of neem cake was significantly superior in disease control (p=0.05) as well as in increasing seed yield (Table 2). These findings are in agreement with those of Shivpuri et al. (1997) and Kapoor et al. (2006) who reported that neem based pesticides were highly effective against *Fusarium oxysporium* f.sp. *pisi*, F. *solani* f. sp. *pisi* and *S. sclerotiorum*. Neem cake possibly enhances the disease control by release of volatile substances during decomposition that induced disease resistance or tolerance on the root surface (Singh, 1983). It is a well-known fact that cakes improve physio-chemical properties of the soil and increase the vigour of the crop by supplying nutrients and promoting anti-microbial populations in the rhizosphere (Sharma and Sharma, 1986). Handoro et al. (2001) reported that application of soil amendments Neemax, FYM and sheep manure to pea crops decreased the disease incidence to S. sclerotiorum and increased seed yield. According to Tripathi et al. (2010), neem cake extract, mustard cake extract and farm yard manure extract reduces the mycelial growth of *S. sclerotiorum*. Sharma et al. (2011) found that the application of mustard cake reduced the stem rot incidence and increases seed yield in cauliflower. Generally, the integration of organic amendments and biocontrol agents have been found to improve the plant health in various crops (Rana et al., 2010; Chawla and Gangopadhyay, 2010; Yadav et al., 2013).

Thus, it was concluded that use of N fertilizer more than 80 kg ha-*1* increases Sclerotinia stem rot while proper application of K fertilizer and addition of organic amendments slowed down the Sclerotinia stem rot in mustard. Hence, balanced fertilizers and organic amendments are necessary for successful management of white stem rot of mustard and improve physio-chemical properties of the soil, increase the vigour of the plant by supplying certain nutrients and encourage anti-microbial population in the rhizosphere apart from providing reasonable disease control in the field experiments.

Conflict of Interest

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors are grateful to the Head, Department of Plant Pathology and Dean, College of Agriculture, S.K. Rajasthan Agricultural University, Bikaner for providing necessary facilities during the course of investigation.

Table 1. Effect of fertilizer levels on disease development of Sclerotinia stem rot of Indian mustard under field conditions.

Treatment ertilizer dose (N:P:K) Kg ha^{-1}	Disease incidence (%)			Disease intensity (%)			Disease control (%)	Seed yield (q ha^{-1})			Increase in seed yield over check (%)
	2007-08	2008-09	Pooled	2007-08	2008-09	Average		2007-08	2008-09	Average	
T_1 60:30:30	36.67 (37.25)*	30.58 (33.35)	33.62 (35.39)	28.33 (32.13)	23.33 (28.84)	25.83 (30.49)	35.42	7.23	7.78	7.51	23.92
T_2 60:30:60	30.47 (33.48)	26.09 (30.70)	28.28 (32.09)	26.67 (31.06)	18.33 (25.30)	22.50 (28.18)	43.75	7.34	8.10	7.72	27.48
T_3 60:60:30	29.93 (33.14)	28.16 (32.03)	29.04 (32.59)	26.67 (31.06)	21.67 (27.70)	24.17 (29.38)	39.58	7.77	8.26	8.02	32.34
T_4 60:60:60	28.06 (31.95)	31.57 (34.17)	29.81 (33.06)	23.33 (28.84)	25.00 (28.84)	24.17 (29.38)	39.58	8.86	8.31	8.59	41.76
T_5 80:30:30	30.91 (33.74)	30.03 (33.21)	30.47 (33.47)	25.00 (29.99)	23.33 (28.84)	24.17 (29.42)	39.58	9.25	8.55	8.90	46.92
T_6 80:30:60	26.02 (30.60)	23.63 (29.06)	24.83 (29.83)	20.00 (26.55)	15.00 (26.55)	17.50 (24.67)	56.25	10.29	10.67	10.48	73.02
T_7 80:60:30	22.31 (28.16)	25.71 (30.44)	24.01 (29.30)	16.67 (27.04)	16.67 (22.59)	16.67 (32.66)	58.33	11.03	11.34	11.18	84.65
T_8 80:60:60	24.42 (29.59)	21.94 (27.91)	23.18 (28.75)	18.33 (25.30)	13.33 (14.75)	15.83 (23.31)	60.42	13.66	13.94	13.80	127.85
T_9 100:30:30	34.48 (35.94)	33.36 (35.27)	33.92 (35.61)	30.00 (33.15)	28.33 (32.13)	29.17 (32.64)	27.08	8.67	8.99	8.83	45.80
T_{10} 100:30:60	36.08 (36.90)	37.13 (37.53)	36.61 (37.21)	31.67 (32.13)	31.67 (35.24)	31.67 (34.22)	20.83	9.01	9.22	9.11	50.45
T_{11} 100:60:30	32.50 (34.68)	38.78 (38.47)	35.64 (36.57)	33.33 (34.22)	33.33 (34.22)	33.33 (35.24)	16.67	9.32	9.38	9.35	54.35
T_{12} 100:60:60	39.01 (38.63)	36.34 (37.06)	37.68 (37.84)	31.67 (32.13)	30.00 (33.20)	30.83 (33.71)	22.92	9.61	9.47	9.54	57.47
T_{13} 100:60:00	52.78 (46.58)	37.80 (37.91)	45.29 (42.24)	41.67 (40.18)	35.00 (36.26)	38.33 (38.22)	4.17	7.61	7.17	7.39	21.95
T_{14} 80:30:00	44.15 (41.62)	41.53 (4011)	42.84 (40.86)	36.67 (37.24)	33.33 (35.24)	35.00 (36.24)	12.50	6.59	6.60	6.59	8.88
T_{15} Control**	64.39 (53.35)	66.92 (54.58)	65.66 (54.11)	43.33 (41.15)	36.67 (37.24)	40.00 (39.20)	-	6.09	6.02	6.06	-
SEm ±	1.02	0.79	0.65	1.27	1.46	0.75	-	0.32	0.39	0.26	-
CD (P=0.05)	2.97	2.28	1.83	2.84	3.27	2.12	-	0.94	1.14	0.72	-

*Figures in parentheses are angular transformed values, **without fertilizers.

Table 2. Effect of soil amendments on disease development of Sclerotinia stem rot of Indian mustard under field conditions.

Treatment	Dose	Disease incidence (%)			Disease intensity (%)			Disease control (%)	Seed yield (q ha^{-1})			Increase in seed yield over check (%)
		2007-08	2008-09	Pooled	2007-08	2008-09	Pooled		2007-08	2008-09	Pooled	
T_1 Mustard cake	1 ton ha^{-1}	34.97 (36.24)*	38.63 (38.41)*	36.80 (37.32)*	30.00 (23.20)	31.67 (34.22)	30.83 (33.71)	24.49	8.19	7.69	7.94	12.98
T_2 Mustard cake	2 tons ha^{-1}	29.58 (32.93)	32.12 (34.48)	30.85 (33.71)	26.67 (31.06)	30.00 (33.20)	28.33 (32.13)	30.61	8.64	8.42	8.53	21.32
T_3 Neem cake	0.5 ton ha^{-1}	25.28 (30.17)	23.49 (28.98)	24.39 (29.57)	20.00 (26.55)	20.00 (26.55)	20.00 (26.55)	51.02	12.43	12.03	12.23	73.98
T_4 Neem cake	1 ton ha^{-1}	21.97 (27.90)	18.70 (25.54)	20.34 (26.72)	15.00 (22.78)	15.00 (22.78)	15.00 (22.78)	63.27	14.19	13.95	14.07	100.18
T_5 Vermicompost	10 tons ha^{-1}	35.92 (36.80)	37.33 (37.63)	36.63 (37.22)	23.33 (28.84)	33.33 (35.24)	28.33 (32.04)	30.61	11.08	10.52	10.80	53.67
T_6 Vermicompost	15 tons ha^{-1}	24.48 (29.63)	32.20 (34.49)	28.34 (32.06)	18.33 (25.30)	28.33 (32.13)	23.33 (28.71)	42.86	12.15	11.90	12.03	71.10
T_7 FYM	10 tons ha^{-1}	41.94 (40.34)	44.18 (41.61)	43.06 (40.99)	33.33 (35.24)	33.33 (35.24)	34.17 (35.75)	16.33	10.90	9.19	10.05	42.94
T_8 FYM	15 tons ha^{-1}	36.19 (36.96)	37.19 (37.56)	36.69 (37.26)	31.67 (34.22)	31.67 (34.22)	31.67 (34.22)	22.45	11.94	11.76	11.85	68.65
T_9 Sulphur	30 kg ha^{-1}	30.99 (33.80)	30.18 (33.29)	30.58 (35.55)	21.67 (27.70)	25.00 (29.99)	23.33 (28.84)	42.86	8.75	8.81	8.78	24.90
T_{10} Sulphur	60 kg ha^{-1}	26.30 (30.81)	23.30 (28.84)	24.80 (39.82)	20.00 (26.55)	21.67 (27.70)	20.83 (27.13)	48.98	9.05	10.19	9.62	36.83
T_{11} Control**		60.34 (50.95)	61.91 (51.87)	61.13 (51.41)	38.33 (38.23)	43.33 (41.15)	40.83 (39.69)	-	6.99	7.07	7.03	-
SEm ±		0.96	1.22	0.78	1.40	1.21	0.60	-	0.40	0.40	0.29	-
CD (P=0.05)		2.84	3.59	2.24	2.68	2.31	1.72	-	1.18	1.18	0.82	-

*Figures in parentheses are angular transformed values, **without soil amendments.

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