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African Journal of Microbiology Research

14 July 2018
ISSN 1996-0808
DOI: 10.5897/AJMR
www.academicjournals.org

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African Journal of Microbiology Research

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Vol. 12(26), pp. 606-615, 14 July, 2018
DOI: 10.5897/AJMR2018.8876
Article Number: 6E0148E57872
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Isolation of bacterial diversity present in medical waste and health care settings in hospitals in Kenya

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Received 2 May, 2018; Accepted 4 June, 2018

Nosocomial infections have impacted great burden in healthcare system and has led to deteriorating health condition and deaths. This study characterizes medically important bacterial diversity, isolated from staff hands, hospital surfaces and wastes in healthcare settings in Kenya during a one year period. Descriptive cross sectional hospital based study design and simple random sampling method was used to collect 246 samples from 10 sections in each hospital, using sterile cotton swabs and processed in the laboratory. Colony morphology and biochemical characterization was also recorded and confirmation of Enterobactericeae using API-20E test for later study of ESBL resistant genes was done. Statistical analysis was done using Microsoft Excel and ANOVA. The study highlighted the presence of *Providentia rettgeri* (21.01%), *Staphylococcus aureus* (18.47%), *Escherichia coli* (13%), other Gram negatives (9.55%), *Pseudomonas aeuriginosa* (9.3%), coagulase negative *Staphylococcus* (CONS) (9.12%), *Serratia marcescens (6.58%), Klebsiella pneumonia* (6.36%), *Proteus vulgaris* (4.03%) and *Enterobacter cloaca* (3%). Most nosocomial infections especially urinary tract infections are caused by these bacteria. It is necessary for hospitals to implement most of the recommended measures in this study to reduce the risk of transmission of pathogens via contaminated hospital surfaces and sites.

Key words: Hospital surfaces, hospital waste, environment, isolation, bacteria, nosocomial infections.

INTRODUCTION

Hospital is the place, which is frequently accessed by the people irrespective of age, sex, race, religion, region and even nationality. The waste generated during entire healthcare activities has higher potential to produce health and environmental hazards than the wastes of other places (Boyce et al., 1997). Hospital acquired infection also called nosocomial infection is an infection acquired in hospital by a patient who was admitted for a reason other than that infection. Nosocomial pathogens are organisms causing diseases that are acquired from the hospital and healthcare environment within few days of admission and are responsible for nosocomial infections (Medubi et al., 2006). The hospital exists as a closed community; it is therefore not surprising that certain microorganisms become predominant and cause diseases (Boyce et al., 1997). The pathogens can be expelled from an infected or colonized patient either through direct contact, aerosols droplets or faeces to the

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environmental surfaces. These pathogens can be contracted by the healthcare workers and even by the patients. Therefore, environmental surfaces in healthcare centers act as reservoir for bacteria and can as well serve as vectors of the bacterial pathogens (Boyce et al., 1997). The risk for nosocomial infections poses a potential patient safety threat (WHO, 2002). These infections are often caused by breaches of infection control practices and procedures, unclean and non-sterile environmental surfaces, and/or ill employees. Kenya like many developing countries experience the problem of getting sufficient medical supply and even worse is the disposal of medical waste. This is due to lack of enforcement of legislation for handling, treatment and disposal. Healthcare surfaces and wastes act as the store house of harmful infectious pathogens. Potential health risk includes spreading of diseases by these pathogens and wide dissemination of antimicrobial resistance genes. Antimicrobial resistance in both pathogenic and commensal bacteria is increasing steadily. Failure of antibiotic resistant bacteria containment is responsible for this expansion. The incidence of infections caused by beta lactam resistant organisms due to the production of various enzymes has increased in recent years. Hospital waste can be hazardous to public health and ecological balance since it can contain various kinds of pollutants such as radioactive, chemical and pharmaceutical wastes and also pathogenic microorganisms (WHO, 2002). In Kenya like many developing countries, data is limited to the number of cases registered in health facilities, like Ibn Sina hospital in Rabat (Bakkali et al., 2015) which highlighted the presence of pathogens in distinct areas of the hospital environment like *Pseudomonas aeuriginosa*, *Staphylococcus aureus*, *Klebsiella* species and various gram negative bacilli. Therefore, the present study was done to evaluate the presence of pathogen bacteria in the environment of Kenyatta National Hospital (KNH) and Kikuyu Mission Hospital (KMH) in Kenya and to characterize them for better management of the hospital environment quality.

MATERIALS AND METHODS

This constitutes bacteriological analysis and it included cultivation of collected samples from various sites or departments in the hospitals, colony morphology study under different selective media, biochemical tests and confirmatory test using API-20E strips. The study site chosen for this study are KNH and KMH situated in Nairobi County (www.knh.or.ke and www.pceakikuyuhospital.org) in which samples were collected from various sites or departments in the hospitals. The study design used in this research was descriptive cross sectional hospital based study with a sample size of 246. Simple random sampling method was used to collect samples from ten departments in each hospital. Sampling was performed during the morning after the regular daily cleaning. Different surfaces and locations were included (door handles, toilet, bathroom knobs, bed rails, cabinet locks and handles, water dispensers taps, tables including operating tables, scrubber surfaces, sink surfaces, theatre equipment surfaces (for example breathing tubes, infusion pump, aspirators), waste bin surfaces, dump site, door handles and knobs and floor surfaces, etc. Basically, 23 to 24 samples were used, at least, for each sampling site, giving a total of 246 samples in each hospital. Samples collected using sterile polyester fibre tipped applicator swabs (Becton Dickson, Basel, Switzerland) were moistened in 2 ml sterile saline solution and rolled several times over a surface area of around 25 cm. They were then put into sterile tubes, tightly capped and labeled appropriately; similarly, sterile swabs were dipped into drainages and treated as earlier stated. They were transported in ice cooler box to the laboratory for processing. Samples were inoculated on various selective and differential media such as MacConkey, Mannitol salt agar (MSA), Eosin methylene blue (EMB), and *Salmonella shigella* agar (SS), using the streaking method. Samples were incubated at 36°C (+/- 1°C) in an incubator for 18 to 24 h (overnight). Visible colonies were further sub cultured and incubated for 24 h at 37°C. Isolation and identification of microorganisms were done according to standard procedures. Bacteria were identified by examination of colonial morphology, on appropriate agar media. Samples from MacConkey media were classified as either lactose fermenters or non-lactose fermenters. Gram staining rapid tests (catalase, oxidase, and coagulase) was done to classify the isolates. Various biochemical tests (indole test, methyl red-Voges Proskauer test, citrate utilization and triple sugar iron test, were performed on the isolates to confirm their identities and aid in bacterial species identification as per the protocol by Tolaro (2005). Results of Enterobactericeae were confirmed using API-20E test (Biomeriux, France) (Baron and Finglod, 1996). Confirmation was necessary for future study in antibiotic sensitivity test (Kirby et al., 1966) and detection of Beta lactamase genes from Enterobactericeae. Data analysis was done using SPSS and ANOVA. Scientific approval of the study was obtained from Kenyatta National Hospital Ethics and Review Committee, while ethical clearance to carry out the study was obtained from KNH and KMH hospitals administration. All procedures were carried out in accordance to the standard biosafety guidelines and waste disposal.

RESULTS

Isolation of pathogen strains from hospitals surfaces and hands

Results from this study revealed that a total of 592 sampling were made from various hospital surfaces and hands of health staffs. KNH had the highest number of positive plates which indicated the presence of bacterial with a total of 197 (80.08%), while KMH had 163 (66.26%). There was no statistical significant increase in the prevalence of contamination in private as compared to public hospitals ($p = 0.38$). Out of those 246 samples, majority of positive plates for the presence of bacterial in both hospitals were from site A (waste from hospital main drainage) with 25 (100%) and site I (orthopedic unit) for KNH with 25 (100%) level of positive growth plates. The least was site C (operation theatre) for both hospitals; KNH had only 6 plates with positive growth out of 25 (24%), while KMH had 9/25 (36%) as indicated in Table 1. Among the 360 positive samples, 471 pathogens strains were isolated, comprising of Gram negative bacteria which were more in most departments, (341, 72.3%) than the Gram positive bacteria (130, 27.7%).

Table 1. Distribution of samples taken from different hospital environments and waste in KNH and KMH.

Sample site	Name of Hospital	Total samples N (%)	Samples positive N (%)	Samples average N (%)
A (waste hospital main drainage)	KNH	25 (100)	25 (100)	25 (100)
	KMH	25 (100)	25 (100)	
B (ICU)	KNH	25 (100)	16 (64)	15.5 (62)
	KMH	25 (100)	15 (60)	
C (Operation theatre)	KNH	25 (100)	6 (24)	7.5 (30)
	KMH	25 (100)	9 (36)	
D (Sterilization area)	KNH	25 (100)	20 (80)	15 (60)
	KMH	25 (100)	10 (40)	
E (Pediatrics ward)	KNH	24 (100)	22 (92)	22.5 (90)
	KMH	24 (100)	21 (84)	
F (Gynecology/obstetric)	KNH	24 (100)	16 (67)	17.5 (70)
	KMH	24 (100)	19 (79)	
G (Internal medicine)	KNH	24 (100)	22 (92)	16 (64)
	KMH	24 (100)	10 (42)	
H (General ward)	KNH	24 (100)	23 (96)	18.5 (74)
	KMH	24 (100)	14 (58)	
I (Orthopedic/surgical unit)	KNH	25 (100)	25 (100)	23 (92)
	KMH	25 (100)	21 (84)	
J (Hospital dump site)	KNH	25 (100)	22 (88)	20.5 (80)
	KMH	25 (100)	19 (76)	
Total	KNH	246	197 (80.08)	73.17
	KMH	246	163 (66.26)	

The distribution of strains according to samples origin

The distribution of strains isolated from surfaces and hands of health personnel and their detection rates were reported in figures and it shows that 471 were isolated from surfaces of various locations in different surfaces and personnel hands. Characterization of strains isolated showed clearly a high prevalence of Gram negative bacteria. Results clearly demonstrate the presence of both Gram positive and Gram negative bacteria with the predominance of *Providentia* species (21.01%), *S. aureus* (18.47%), *Escherichia coli* (12.95%), other Gram negatives such as *Roultella ornithylitica, Ochrobactrum anthropic, Pantoea* species (9.55%)*, Pseudomonas* species (9.34%), coagulase negative *Staphylococcus* (CONS) (9.12%), *Serratia* species (6.58%), *Klebsiella* species (6.36%), *Proteus* species (4.03%), and *Enterobacter* species (2.54%) as shown in Table 2. Most of the frequently isolated bacterial included *E. coli* in waste water main drainages (A) while *Providentia*, *Pseudomonas*, *Serratia* and *Klebsiella* species were the most abundant in ICU, while *S. aureus* and other coagulase negative Gram positives were abundant in ICU (B), general ward (H). Moreover, the percentage of detection of Gram positive and Gram negative bacteria isolated at different locations is summarized in Figure 1. For both Gram negative and Gram positive bacteria, the main infected surfaces were the door handles and nurses hands surfaces representing the major reservoirs of pathogens. Moreover, Gram negative bacteria were predominant in sinks and waste waters, whereas Gram positive bacteria prevail in samples taken from door handles and nurses hands surface as reported in Table 2.

Pseudomonas species was most abundant in sinks (20.4%) followed by operation table (18.18%) (Table 2). In operation table (26.66%), *Klebsiella pneumoniae* was most abundant followed by door handles (20%). *E. coli* was found mostly in nurses' hands surface (NHS) (22.9%), followed by nurses' staff table (NST) (19.6%). *Proteus vulgaris* was found in stretchers and elevator

Table 2. The occurrence of bacterial isolates according to samples origins.

Sample source	Sample size	*Pseudomonas* spp.	*Klebsiella* spp.	*E. coli*	*Proteus* spp.	*Enterobacter cloaca*	*Providentia* spp.	*Serratia* spp.	Other Gram negatives	*S. aureus*	Other coagulase negative staphs	Total number of bacteria in each location
NHS	38	0	0	14	0	6	5	0	2	19	4	50
NST	37	0	5	12	2	1	7	0	6	12	1	46
DH	38	0	6	10	0	2	14	0	1	15	11	59
TS	38	7	0	0	1	1	11	4	3	11	2	40
OT	37	8	8	8	2	0	8	1	2	6	1	44
Sink	38	9	2	0	3	0	11	5	6	0	0	36
Stretcher	38	0	0	9	5	1	13	1	3	3	2	37
Floor surf	38	7	1	0	1	0	11	3	1	3	1	28
EB	38	4	0	5	5	1	2	0	1	3	17	40
WW	38	6	4	0	4	0	7	7	9	0	0	35
DS	38	2	2	0	0	0	5	7	8	0	2	26
BR	38	0	3	1	1	1	2	2	1	6	1	18
CB	38	0	0	2	0	0	3	1	2	7	1	16
Total	492	44	30	61	19	12	99	31	45	87	43	471

buttons while *Enterobacter cloaca* was abundant in nurses' hands surfaces. Species of *Providentia* were mostly found in door handles at 14.14%, *Serratia* species were most abundant in waste water samples and door surfaces at 22.58% in both cases. Other Gram negative isolates were found in waste water and door surfaces. *S. aureus* was abundantly found in nurses' hands surface in about 21.83%, while other coagulase negative *Staphylococcus* was most abundant in elevator buttons (39.53%) and door handles in 25.58% occurrence (Table 2).

DISCUSSION

In this study, it was clearly demonstrated that the hospital with the most positive plates indicated higher contamination. More contaminated plates were observed in this study from the public hospital, which is slightly more but not significant in the private hospital. This is a reflection of the practices in these establishments and may be attributed to improper or insufficient treatment of the wastes before disposal (Sridhar and Olajumoke, 2003). It can be explained by the fact that majority of people are low income earners in this part of the country and ordinary working citizens who tend to patronize the public hospital because of lesser medical charges as compared to the private hospital where charges are higher (Sridhar and Olajumoke, 2003). Moreover, the public hospital pre-treats their wastes before disposal. Occurrence of bacterial isolates generated from different departments, wards and waste effluent sites at both hospitals is revealed in the study. The department with the highest level of contamination was A (main drainage waste water) in both hospitals (100%) with all the plates indicating positive. The department, with the least contamination was site C (operation room) probably because of the level of efficiency in use of disinfectants and sterilization in the operation room as confirmed by research done by Moges et al. (2014) in Ethiopia, who found the operation room with lesser bacterial contamination as compared to other hospital units.

There were significant differences in the number of bacterial isolates in the orthopedic/surgical departments in both hospitals. Several factors may contribute to this; the difference in quality of the ventilation system, the difference in cleaning procedures and the difference in traffic in these areas as revealed by Moges et al. (2014). It was also noted that about 20% of hospital dump site waste samples in both public and private hospital departments, showed no growth at all. This could probably be due to the nature of the organism or the effect of possible pre-treatment given to wastes as researched by Sridhar and Olajumoke

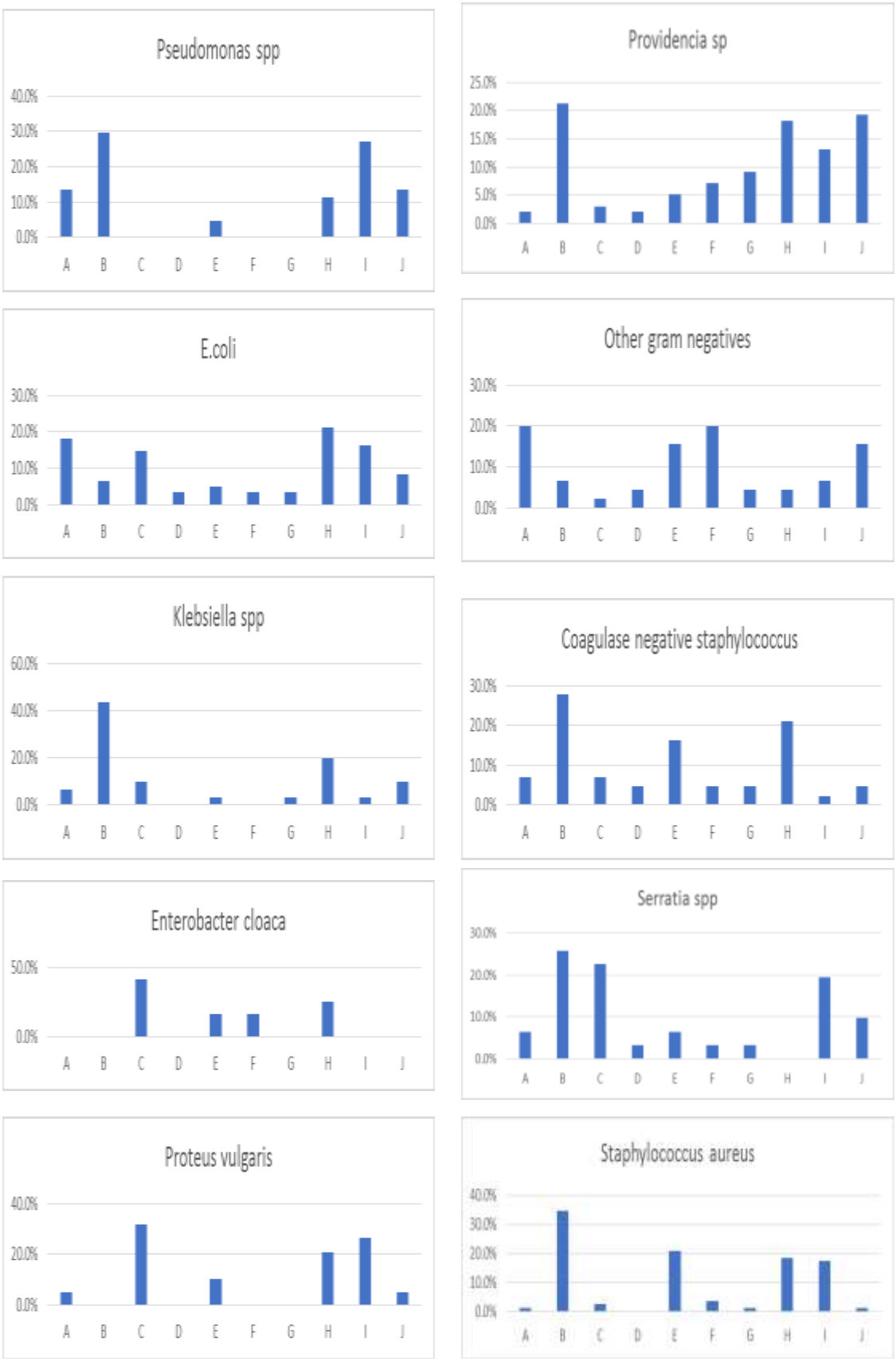

Figure 1. Frequency of each bacterium isolated from each sample point (hospital departments).

(2003).

Many of the bacterial isolates found in the waste samples reported here are also known to cause hospital acquired or nosocomial infections (from the records of Hospital, 2014). It is however believed that hospital surfaces and medical wastes are the least possible source of a hospital-acquired infection. This study confirms this point, but cautions that in the communities, these wastes can be a health risk. There is need to manage them properly and also to train waste generators and handlers on safe work practices during collection, storage and transportation. Both lactose fermenting and non-lactose fermenting bacteria were isolated and identified. From the result, more Gram-negative organisms [especially members of the Enteriobacteriaceae 341 (72.3%)] were isolated than Gram positive organisms 130 (27.7%). This agrees with research done in Markudi, Benue State, Nigeria by Omoni et al., 2015, who revealed that more Gram negatives were present than Gram positive in samples collected from hospital settings. In the study, *Providentia* spp. (21%) and *E. coli* (13%) were among the Gram-negative bacteria while *S. aureus* was amongst the Gram-positive bacteria (18%); among the majority isolated from the samples collected from the current study. The high frequency of pathogenic bacterial in this study may be due to high admission of cases with bacterial infections, which is common in developing countries like Kenya (Hospital records). This study also conforms to the work of Anitha and Jayraaj (2012) who reported *E. coli* a Gram negative bacteria as the predominant organism in hospital wastes and also there was presence of Gram-positive isolates such as *Bacillus subtilis* and *S. aureus* in biomedical wastes collected in a public and private hospital in Coimbatore, India. Characterization of pathogen strains showed the predominance of Gram negative bacteria from surfaces and hands of health professionals. Gram positive pathogens such as staphylococcus strains show much higher transmission rates as compared to Gram negatives. That could be explained by diminished survival time of Gram negatives in the environment (Gastmeier et al., 2006). Infact, Gram negative bacteria other than *Acinetobacter* species (Wendt et al., 1997), survive on dry surfaces for few hours only, while the survival time can be several days for *Staphylococci* (Hirai, 1991).

In this study, the pathogenic bacterial isolated from the medical waste and hospital environment included *Providentia* spp. (21%), *S. aureus* (18.5%), *E. coli* (13%), *Pseudomonas* spp. (9.3%), and other coagulase negative *Staphylococci* (9.13%), *Serratia marcescens* (6.6%), *Klebsiella* spp. (6.4%), *Proteus vulgaris* (4%), and *E. cloaca* (3%) among others. Some of these isolates have been reported by earlier researchers (Yagoub and Agbash, 2010). Similar reports by Ekhaise and Omavwoya (2008) in Benin hospital and Vichal et al. (2011) showed that the bacterial genera, *Klebsiella, Pseudomonas* and *Serratia* were the most frequently distributed isolates in the hospital wastewater. In another study, *Pseudomonas* spp. was found to be the most prevalent by 20.7% (Ashfaq et al., 2013). In the study conducted by Oyeleke and Istifanus (2009), the most predominant pathogens isolated from hospital wastes were *Bacillus* and *S. aureus* (80 to 90%); however, findings by Oyiasogie et al. (2010) showed that *P. aeruginosa* was among the highest Gram negative organism isolated from hospital waste accounting for about 25.00% overall of all the isolates. In a study carried out in Erbil city, Rhizgari by Aziz et al. (2014) revealed that *E. coli* was mostly isolated (100%) from a hospital wastewater. It is widely accepted that these pathogens are the major cause of hospital acquired infections which is in agreement with this study that *E. coli* strains were obtained (13%) from both hospitals being second to *Providentia* spp. A similar observation was made in hospitals in Ethiopia (Yismaw et al., 2010) where it was reported to be among the most frequent isolates. The high occurrence of *Providentia* spp. and *E. coli* isolates in these samples could be attributed to poor hygienic conditions in the hospitals studied and the conditions in other hospitals are not different as the country lacks adequate number of healthcare facilities (Yismaw et al., 2010). These results in overcrowding in the few hospitals available and hence the unhygienic conditions.

In the present study, *Salmonella* and *Shigella* species showed positive growth in the *Salmonella-Shigella* agar but were not positively identified from the surface swabs and wastes collected in hospitals in the confirmatory tests using API-20E. This may probably be due to the nature of the organism; viable but non-cultivable or the effect of possible pre-treatment given to wastes. Dudley et al. (1980) also reported variety of pathogenic bacterial in sewage sludge; however, *Shigella* spp. were not detected in their study due to low sensitivity of enrichment procedure and high temperature which decreased its survival in their study.

Providentia spp. are members of Enterobacteriaceae and uncommon cause of infections, although among the species *Providentia rettgeri* and *Providentia stuartii* are the most common causes of infections, especially urinary tract infections (UTIs) and bacteremia in hospitalized patients or nursing care facilities. A few studies have been published on the subject. In a review done by Kim et al. (2007), *Providentia* spp. incidence was 0.16%; our study confirms that *Providentia* spp. is uncommon and that the incidence rate in this study was higher than that by Kim et al. (2007). *Providentia* spp. are the most frequently isolated from elderly patients or patients with urinary catheters. The reason for the variable incidence of *Providentia* is not apparent, but the types of patients and institution might contribute to such a difference. There were significant number of bacteria especially *Providentia* spp. on the floors of various areas in the operating suite; several factors may contribute to this.

First, the difference in quality of the ventilation system; secondly, the difference in cleaning procedures; thirdly, the difference in traffic in these areas. We consider the major contributing factor to be the difference in cleaning procedures. On the basis of researchers' observations, it is recommendable that, there should be regular use of disinfectant in cleaning the operating room floor after every operation. There are several reports on the use of disinfectant on cleaning the floors. Rutala and Weber (2014) reported a significant reduction in floor bacteria with the use of a germicidal detergent. He also reported that the floors in the inner zones of the operating suite cleaned with disinfectant showed low level of bacterial contamination.

S. aureus strains were the second most frequently isolated bacterium (18.5%) from this study. This finding is similar to Yagoub (2010) report who found that Gram positive bacteria such as *S. aureus* in particular as most pre-dominant bacterium in their study. These results were also observed by Perwaiz et al. (2007) who obtained an isolation rate of *S. aureus* at 13% and the second most frequent pathogen. It has been frequently isolated from nurses/doctors' hands including nurses' staff table. Several studies have reported the importance of frequent and adequate hand washing to reduce rates of hospital acquired infections (Rupp et al., 2008), showing that hands regularly acquire bacterial pathogens responsible of nosocomial infections and can survive on dry surfaces for several weeks (Frost and Sullivan, 2010). Elevator buttons had more coagulase negative *Staphylococcus* spp. (9.3%) than other surfaces; this could be due to the fact that they are touched repeatedly by ungloved hands by multiple individuals who will later go on to contact patients colonized by bacteria that were not pathologic in most case but overall prevalence rate exceeded sink surfaces. Furthermore, if hand hygiene practices are suboptimal, microbial colonization is more easily established and/or direct transmission to patients or a fomite in direct contact with the patient may occur as concluded by Allegranzi and Pittet (2009). It has been reported that organisms are capable of surviving on hands of health care workers for at least several minutes following contamination (Allegranzi and Pittet, 2009); hence, the necessity of a hand washing facility at most points in a health care institution. Thus, risk of infection is high in individuals occupationally exposed to wounds or wound dressing indicating a need to screen individuals in hospitals for risk exposures and infections, to avoid outbreak and cross infections in hospitals for risk exposures and infections as described by Perwaiz et al. (2007) in their research work. *Staphylococcus* strains has been incriminated in various diseases such as post-operative infections, urinary tract infections, skin diseases, respiratory infections and food poisoning (Murray et al., 1995; Buchanan and Gibbons, 1974). The *S. aureus* strains were mostly isolated from hand swab of the nurses from this hospital and were almost the same as compared to the earlier report of Boyce (2007) and Ekrami et al. (2011). The high prevalence of the *S. aureus* from hand swabs and door handles in this work might be as a result of inadequate hand hygiene and this could be one of the attributing factors of the distribution of the pathogen in the hospital environmental surfaces as reported earlier by Olalekan et al. (2011). The low prevalence rate of *S. aureus* on beddings and bed rails in this hospital is not in agreement with 100% prevalence on bedrail as reported by Boyce (2007). Also, 26% of *S. aureus* reported on door handle by Carvalho et al. (2007) is higher to the prevalence rate of the *S. aureus* on door knob/door handle of 17% from these hospitals in the current study. The prevalence rate of 16% of *E. coli* on door knobs/door handles confirms the early report of Nworie et al. (2012) from some parts of Abuja metropolis that the contamination of door knob/door handle can be as a result of poor hand hygiene after using toilet. In addition, results from this investigation, recommends that materials contaminated with patients' secretions, such as saliva, sputum and mucus, should be cleaned with disinfectant or discarded. This is especially true of patients' pillows, which are usually contaminated with secretions from mouth, nose and trachea. In one instance, *S. aureus* was found in a pillow (beddings and bed rails category). Contamination of the operating light in the operation theatre was reported by Husein et al. (2001). Since operating lights are cleaned daily with disinfectant, it was not found holding any contamination. It was found that *S. aureus* and coagulase-negative *Staphylococci* species was the major species contaminating floors and other surfaces in the operating rooms. *Staphylococci* are usually human in origin and point to the restriction of traffic in operating rooms,

Pseudomonas strains were obtained in this study with a prevalence rate of 9.3%. Similar prevalence rate of 9.3% was reported by Srinivas et al. (2015) in Andhra Pradesh, India. In comparison, higher prevalence rate of 32.1 and 20.3% was reported by Rajat et al. (2012), in Gujarat, India, respectively. This varied prevalence of *P. aeruginosa* in different places may be attributed to the type of swab received for examination, type of hospitals and geographical positions. It is widespread in natural environments and it is an opportunistic pathogen for humans lead to a broad spectrum of disease such as urinary, burn, respiratory infections and septicemia. *Pseudomonas* spp. is one of the most common isolated pathogens from hospitalized people and that it thrived on moist surfaces as also confirmed by Nagoba et al. (1997), heightening the risk of infection for patients with catheters or ventilators; from its commonly found wet places it reaches sick patients admitted in hospital and causes a variety of serious infections in hospitalized patients with impaired defenses. It causes serious infections such as bacteremia pneumonia, sepsis, burn wound infections and meningitis. In our studies, the

highest number of *Pseudomonas* spp. was isolated from sinks (20.45%), floors and waste water drainages. The isolation of *Pseudomonas* spp. from the sinks confirms the report of Udeze et al. (2012) that sinks are the most common place in hospital environment where *Pseudomonas* spp. are predominantly found. Sinks are the most common article of contact by the people. It is therefore and not surprising that it also gave very high *P. aeruginosa* isolates since people with wet hands (water or sweat) may easily come into contact with it. The places with least number of isolates were the bedrails, stretchers, nurses staff tables, etc., and these are places that are likely to be dry most of the time in the hospitals. Also, the prevalence rate of *Pseudomonas* spp. on operation table of the hospitals in the current study hospital was still higher (18.18%) than a work reported by Pal et al. (2010) with about 9.6% of the pathogen was isolated from operation table in a hospital in India. The presence of this pathogen on operation table can contaminate open wounds of the patients in course of the operation.

Serratia spp. also isolated in the current study is an opportunistic, Gram negative pathogen which belongs to family Enterobacteriaceae. It was originally considered as non-pathogenic, but it was discovered that ICU are often involved in the epidemics of the colonization and the infection with *Serratia marcescens*. The important reservoirs in epidemics are the digestive and respiratory tracts. The current study of *S. marcescens* was found mostly in ICU with the highest population sinks, door surfaces and waste in ICU and this was confirmed by research done by Mlynarczyk et al. (2007); it accounts for only 1 to 2% of the nosocomial infections and caused by instrumentation (urinary catheterization or placement of the endotracheal tube for ventilation).

Proteus mirabilis was isolated in some of the units in this study with a prevalence rate of 4%. This species is implicated in many clinical conditions. In other studies, it was also isolated in some residential areas not close to any health care facility (Giske et al., 2008), which suggests that it is present in the general environment.

Results from the current study reveals that bacterial isolates found in the hospital surfaces, waste samples and dumpsites reported here are also known to cause hospital acquired or nosocomial infections (from the records of KNH and KMH, 2015). Bacterial pathogens may develop in wastes undergoing decomposition in soils that suffer from environmental pollution as a result of indiscriminate disposal of pollutants. These bacterial pathogens, when increased in population, pose great risk to human health (Onweremadu et al., 2009). Soil-transmitted pathogens play an important role to the emergence of community-acquired infections, contributing to the burden of communicable disease morbidity and mortality. The bacterial pathogens in the soil and wastes are not considered as public health concern (Santamaria and Toranza, 2010). Little information is available on the types of microorganisms associated with and isolated in waste dumpsite soil consequently; comprehensive assessments on pathogenic organisms must be established to build local knowledge about public health issues and trends in hospital infections and waste management. Other Gram negative bacteria species for example, *Pantoea* spp. was also isolated (12 isolates) in this study. Actually, several studies have reported the association of this germ with nosocomial infections (Liberto et al., 2009). The fungi that were morphologically identified in this study were suspected to be *Fusarium* and *Penicillin* spp.; their spores are common in environment and are responsible for allergic infection of human and animals (Thurston and Cysewsk, 1979). It is however believed that hospital wastes are the least possible source of a hospital-acquired infection. This study confirms this point, but cautions that in the communities, these hospital surfaces and wastes can be a health risk. There is need to manage them properly and also to train waste generators and handlers on safe work practices during collection, storage and transportation. In conclusion, there is need to study antimicrobial resistance rate in hospitals and the possible dissemination of resistant bacteria in the inanimate surfaces or the hands of health professionals reinforce the need for knowledge and control of the sources of pathogens in the hospital environment. The evaluation of the environmental role in the acquisition of healthcare associated infections is needed to collaborate with infection control committees. The establishment of a control system is also required in hospitals for the reduction of length of stay, costs and morbid-mortality. Such a surveillance system should continuously report the prevalence of microorganisms and their resistance pattern to hospital wards; this information will be used in defining policies for control of hospital environments, and building awareness especially in Kenyan hospitals where antimicrobial prescription is sometimes inappropriate.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors acknowledge fundings from National Council of Science, Technology and Innovation.

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Vol. 12(26), pp. 616-622, 14 July, 2018
DOI: 10.5897/AJMR2018.8812
Article Number: C177FE257874
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Fungal endophytes isolated from the leaves of a medicinal plant, *Ocimum sanctum* Linn and evaluation of their antimicrobial activities

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Received 3 February, 2018; Accepted 7 June, 2018

The endophytic fungi isolated from leaves of *Ocimum sanctum* Linn. of different ages were examined for antimicrobial activity. The agar plug diffusion assay was used for primary screening. A total of 148 fungal endophytes were successfully isolated and cultured but only 134 of them (90.5%) exhibited inhibitory activity towards at least one test microorganisms. Moreover, the colonization rate indicated that the old leaves were frequently and densely colonized by endophytes. The results suggested that healthy leaves at older stages of growth can be a potential source for the isolation of endophytic fungi with antimicrobial properties. The ethyl acetate extract prepared from the fermentative broth exhibited better antimicrobial activity and it suggested the antimicrobial activity of the isolates was affected by the culture medium. A better antimicrobial activity was observed in the yeast extract sucrose broth as compared to malt extract broth. Significant improvements in the antimicrobial activity of the crude extract were observed after addition of water extract of the host plant in the culture medium.

Key words: *Ocimum sanctum* Linn., endophytic fungi, antimicrobial activity, host plant extract.

INTRODUCTION

Endophyte is a group of endosymbiont, mostly filamentous fungi (Tiwari et al., 2010) which inhabits a unique biological niche and is categorized as highly diverse, polyphyletic group of microorganisms that are capable of colonizing plants tissues asymptomatically without initiating any disease or overt negative symptoms. Endophytes are believed to benefit host plants by preventing them from colonization of pathogenic microorganisms. The endophytic fungi are reported to produce antimicrobial compounds, having unique genetic and biological systems that may be involved in host-endophyte relationship (Strobel, 2003). Many endophytic fungi have been proven to have the ability to produce novel secondary metabolites to overcome pathogenic invasion. Interestingly, the host-endophytic relationship is found to be complex and varies in different hosts or microorganisms (Tan and Zou, 2001; Pullen et al., 2002).

The study of bioactive compounds and secondary

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metabolites from plants have been extensively discovered for years, based on their significant therapeutic effects practiced in traditional medicines (Nascimento et al., 2000; Souza et al., 2012). *Ocimum sanctum* Linn, which belongs to family Lamiaceae is one of the important herbs due to their therapeutic potential (Agarwal et al., 2013). It is widely distributed in tropical parts of Asia, Africa, Central and South America (Pushpangadan and Bradu, 1995; Balyan and Pushpangadan, 1998; Saha et al., 2010). Its leaves have been reported to be traditionally used for getting relief from common cold, bronchitis, cough and digestive problems (Pattanayak et al., 2010). The leaves contain a bright yellow volatile oil that possess various activities including antiasthmatic, antisuppressant, analgesic, antifungal, antibacterial and antimicrobial properties (Shekhawat and Shah, 2013) and including insecticidal properties (Azevedo et al., 2000; Manjula et al., 2002). However, the medicinal values of its endophytes have not been fully investigated. The objective of this study was to isolate endophytic fungi from the leaves of the medicinal herb and to study the antimicrobial activity of the isolates on various test microorganisms. In addition, the study also aimed to ascertain the effects of plant extract in the culture medium on antimicrobial activity.

MATERIALS AND METHODS

Collection of plant material

Healthy plant leaves samples at different maturity stages with no visible symptom of disease were carefully selected and hand-picked from *O. sanctum* Linn. which was planted in Pasir Putih, Kelantan, Malaysia (Latitute 5.832327812453 and Longitude 102.38352936481) on 27th April, 2014 and were stored in separate clean zip lock plastic bags. The collected samples were kept at 10°C during transportation and processed within 4 h after collection. The leaves were washed under running tap water and dried in room temperature (30±2^{o}C) until constant weight obtained.

Estimation of chlorophyll content

Fully expanded leaf samples were selected at different growth stages: young, mature, senescent and old. The chlorophyll content was measured in triplicate using SPAD-502 meter (Konica-Minolta, Japan) around the midpoint near the midrib of each leaf sample. The average SPAD meter values were calculated to estimate the amount of chlorophyll present in the leaf.

Surface sterilization

The isolation of endophytic fungi was conducted using method described previously by Okuda et al. (2005) and Tong et al. (2011) with some modifications. At first, the surface sterilization was performed on the leaf samples that were air-dried after washing thoroughly under running tap water. They were then soaked in 70% (v/v) ethanol for 1 min and rinsed with sterile distilled water. Subsequently, they were immersed in 1% (v/v) sodium hypochlorite for 1 min followed by rinsing three times with sterile distilled water. The step was repeated for different immersion times viz. 0, 5, 10, 15 and 20 min with 5 min interval for each step, in order to determine the effectiveness of surface sterilization process for removing epiphytic fungi. The experiments were done in triplicates.

Vitality test

To assure the effectiveness of the surface sterilization, the vitality test (Petrini, 1998) was then carried out where the top and bottom parts of each leaf samples were printed onto potato dextrose agar (PDA) plate. The plates were incubated at 30°C for 7 days and the viability of the epiphytic microorganisms was observed. The samples that showed no visible epiphytic microorganisms on PDA plates were further examined for surface imprint test.

Leaf imprint test

The efficacy of the immersion procedure was examined by leaf surface imprint test to optimize the time of immersion (Tong et al., 2011). The sterilant-treated leaf samples at different immersion time were imprinted onto PDA plates and then incubated at 30°C for 14 days. The viability of the epiphytic microorganisms was observed after the incubation period. The sterile leaf samples with shortest immersion time (that showed no visible growth of epiphytic microorganisms on PDA plates) were selected for further isolation procedures.

Isolation of endophytic fungi from leave samples and storage

Preparation of plant powder and plant extract

Healthy disease free leaves samples were initially washed thoroughly under running tap water to remove dust and debris on the surface of leaves. They were then rinsed once in sterile distilled water and allowed to dry at room temperature (30±2^{o}C) for a week before transferred to 60°C oven until a constant weight was obtained. The dried leaves samples were then ground into fine powder form. The leaf powder was then kept in a desiccator to avoid moisture which can cause nutrient loss and fungal contamination prior to use. As for plant extract, 5 g of powdered leaf materials were added into 1000 mL distilled water and boiled for 30 min. The extracts were then filtered using Muslin gauze followed by Whatman No. 1 filter paper (Tong et al., 2011). The filtrate was then used for culture media preparation.

Preparation of growth agar media

Six types of growth agar media which were based on potato dextrose agar (PDA) and malt extract agar (MEA) were used. The growth agar media were plain PDA, PDA plus host plant powder (PHP), PDA plus host plant extract (PPE), plain MEA, MEA plus host plant powder (MHP) and MEA plus host plant extract (MPE). All media were autoclaved at 121°C for 15 min and supplemented with 0.2 g/L chloramphenicol to suppress the growth of bacteria.

Isolation of endophytic fungi

The leaves samples were aseptically cut into small pieces (5 x 5 mm^2) after a final washing with sterile 0.5 g/L Tween 80 solutions. They were placed (3-4 leaf pieces per plate) onto the isolation media: PDA and MEA with and without the addition of dried powdered plant materials (10 g/L) or host plant extract. Chloramphenicol (0.2 g/L) was added to suppress the growth of

bacteria. The inoculated plates were incubated at 30°C and observed for the sign of endophytic fungal growth every day until the growth of hyphal tips on the media detected. The hyphal tips were aseptically cut into small fragments and transferred onto fresh agar media. The endophytic fungi were repeatedly cultured to ensure the genetic purity. The pure cultures were grown on slant agars and kept at 4°C with proper labelling. The cultures were deposited at the culture collection of the Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia.

Evaluation of antimicrobial activity

Preparation of test microorganisms

Twenty one test microorganisms provided by the Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia were used in this study including 7 Gram-positive bacteria (*Bacillus cereus* ATCC 10876, *Bacillus subtilis* IBRL A3, Methicillin-resistant *Staphylococcus aureus* ATCC 33591, *Staphylococcus aureus*, *Pseudomonas aeruginosa* ATCC 27844, *Streptococcus mutans* and *Streptococcus agalactiae*), 6 Gram-negative bacteria (*Klebsiella pneumoniae* ATCC 13883, *Shigella boydii* ATCC 9207, *Escherichia coli* IBRL 0157, *Salmonella typhimurium, Yersinia enterocolitis* and *Proteus mirabilis*), 3 yeasts (*Candida albicans* IBRL 1, *Candida utilis* IBRL 1 and *Cryptococcus* sp. IBRL 1) and 5 fungi (*Microsporum fulvum* IBRL SD3, *Trichophyton rubrum* IBRL SA1, *Aspergillus fumigatus* IBRL S1, *Fusarium solani* and *Rhizopus* sp.). The bacterial cultures were subcultured every two weeks on fresh nutrient agar (NA) slants and incubated at 37°C, whereas the yeasts and fungal cultures were subcultured every four weeks on the fresh potato dextrose agar (PDA) slants and incubated at 37°C for yeasts and 30°C for fungi. All the cultures were then kept at 4°C until further use.

The inocula of bacteria and yeast were prepared by transferring two single pure colonies into 5.0 mL of 0.85% sterile physiological saline (w/v) and mixed well to obtain cell suspension. The turbidity of the bacterial and yeast suspension were adjusted to match 0.5 McFarland standards (approximately 1 x 10^8 CFU/mL for bacteria and 1 x 10^6 CFU/mL for yeast). To obtain the desirable inoculum size as suggested by CLSI (2004, 2006), further dilution with 0.85% (w/v) sterile physiological saline was carried out. As for inocula of test fungi, 10 ml of 0.85% (w/v) sterile physiological saline was added into the agar slant containing the 7 days old culture. The slant was shook thoroughly until most of the fungal spores were suspended in the sterile physiological saline. The density of the spore suspension (approximately 1 × 10^6 spores/mL) was counted using a haemocytometer slide (Neubauer, Germany) under a light microscope. Further dilution with 0.85% (w/v) sterile physiological saline was carried out in order to obtain the desirable inoculum size as recommended by CLSI (2004, 2006).

Agar plug diffusion assay

Primary screening of antimicrobial activity of the endophytic fungal isolates was studied by adopting the modified agar plug method proposed by Mohanraj et al. (2011). Agar plugs (1 cm in diameter and 4 mm thickness) were prepared by inoculating the endophytic fungal cultures onto PDA agar plate supplemented with host plant powder and incubated at 25°C for 20 days before cutting them using a sterile cork borer. The agar plugs were then placed on the Muller Hinton (MH) agar seeded with test microorganisms and the plates were initially kept overnight at 4°C to allow diffusion of bioactive compounds and subsequently incubated at either 30°C (for fungi) or 37°C (for bacteria and yeast). Ketoconazole (30 µg/mL) and chloramphenicol (30 µg/mL) were used as positive controls for bacteria/yeast and fungi, respectively. The inhibition zone formed around the endophyte agar plugs were measured after incubation for 72-96 h for fungi, and 24-48 h for yeasts or 16-18 h for bacteria.

Statistical analysis

The Kruskal-Wallis H test was carried out to compare the endophytic fungi assemblages of different leaf age maturity and different culture media. Mann-Whitney U test was performed to determine pair wise comparison if the result from Kruskal-Wallis H test were significant. Two null hypotheses were proposed: (1) There is no significant difference between the numbers of endophytes isolated from different leaf age maturity stages. (2) There is no significant difference between number of endophytes isolated from different culture media. Statistical significance was assumed at the 0.05 levels (p<0.05)

RESULTS AND DISCUSSION

Estimation of chlorophyll content

Chlorophyll content and photosynthetic pigment assessments in every leaf with different growth stages is a very important indicator to measure the leaf senescence since chlorophyll will be lost due to the environmental stress (Yamamoto et al., 2002). The estimation of chlorophyll content using SPAD 502 meter is performed to select the best accurate stages of leaf maturity (Netto et al., 2005). Table 1 shows relative chlorophyll content of *O. sanctum* leaves at different growth stages. The content of chlorophyll increased from young (22 SPAD unit) where the leaf color was light green to mature leaf with the highest chlorophyll content (35 SPAD unit) with the dark green of coloration. The chlorophyll content reduced when the leaf was old (30 SPAD unit) with light green-yellow coloration and at the senescent stage, where the chlorophyll content was 15 SPAD unit with green-yellow coloration. Zhang et al. (2006) reported that chlorophyll content of a leaf was maximum at 60 days old and continued decreasing at 90 and 120 days. The findings obtained from the current study showed that the chlorophyll content was low at a young stage and achieved its highest content at mature stage. However, during old and senescent stages, the chlorophyll content decreased and this could be due to degradation process (Silla et al., 2010). Kamble et al. (2015) reported that chlorophyll content is higher in old mature leaves as compared to the young ones. Determination of chlorophyll content is important to group the leaves in various stages of growth: young, mature, old and senescent.

Plant materials and isolation of endophytic fungi

The selection of plant materials and sampling area are crucial, and they are the key determining factors for successful isolation of endophytes with pharmaceutical

Table 1. Colour and relative chlorophyll content of *O. sanctum* leaves at different growth stages.

Leaf maturity	Young	Mature	Old	Senescent
Colour	Light green	Dark green	Dark green + Yellow	Green +Yellow
Diameter of leaves (mm)	20±0.5	40±1.2	50±1.5	52±0.7
Chlorophyll content (SPAD unit)	22	35	30	15

Table 2. Optimization of immersion time in sodium hypochlorite of different leaf stages of *Ocimum sanctum* Linn.

Leaf maturity stages	Sodium hypochlorite immersion time (minutes)					
	0	2	4	6	8	10
Young	+	-	-	-	-	-
Mature	+	+	-	-	-	-
Old	+	+	-	-	-	-
Senescent	+	+	+	-	-	-

(+); Presence of epiphytic fungi, (-); absence of epiphytic fungi.

potentials. The maturity of the host plants, and environmental factors such as rainfall and atmospheric humidity may affect the diversity of the isolates (Chareprasert et al., 2006). In this study, only the leaves were selected to be used to isolate the endophytic fungi since in traditional medicine, the leaves were reported to possess various pharmaceutical activities including antibacterial, antifungal and insecticides (Chowdhary and Kaushik, 2015). Yu et al. (2010) suggested that the healthy leaves, showing no disease symptoms and cultivated at pesticide free environment but surrounded by infected plants, are more likely to be selected. The endophytic fungi were most prevalent in the leaves and this could be due to their thin cuticle layers (Hiremath et al., 1996). Tong et al. (2011) revealed that the leaves of the host plant were the most prevalent part to be penetrated by endophytic fungi and they found that about 67% of isolates were successfully isolated. Besides, the large leaf surface areas (5 cm in length and 2 cm in width) and the thin cuticle layer could provide more surface area for endophytic fungal penetration and colonization. It is also believed that the role of leaf as a plant photosynthesis area might induce the density of endophytes.

Leaf surface sterilization

Surface sterilization can be performed using sodium hypochlorite, ethanol, formaldehyde, hydrogen peroxide or even acidic electrolyzed water (Okuda et al., 2005; Tong et al., 2011). This is crucial especially for the surface sterilization of fragile samples such as leaves. Some of the sterilants such as sodium hypochlorite and ethanol can cause the less robust samples, not accessible to the propagation of the microbial endophytes (Charepresert et al., 2006). Since the study of surface sterilization on endophytes is method-dependent, therefore, different hosts and plant tissues required different sterilization time. The immersion time of the samples in sodium hypochlorite solution were optimized to ensure elimination of all the epiphytes. Table 2 shows the immersion time used in this study where 1% (v/v) sodium hypochlorite was used. Young leaves need 2 min to eliminate the epiphytic fungi whereas mature and old leaves need 4 min of immersion time. Furthermore, senescent leaves need a longer immersion time (6 min) in order to remove all the epiphytic fungi. The results revealed that the young leaves showed less growth of epiphytes while senescent leaves showed high growth of epiphytes. The results obtained were in agreement with Ibrahim et al. (2014) who reported that the young leaves need less immersion time to eliminate epiphytes. Tong et al. (2014) postulated that the optimization of immersion time of plant sample sterilization is very crucial to ensure the success of the isolation works because a short immersion time might not be sufficient to remove all endophytes from the host plant samples whereas a prolonged immersion time could cause significant damage to delicate samples such as flower and leaf which further affect the viability of endophytes residing in host plant sample (Strobel and Daisy, 2003). Oyebanji et al. (2009) stated that the use of sodium hypochlorite for surface sterilization is enough to remove the epiphyte fungi, dirt or even other contaminants on leaf samples and these procedures are the most frequent choice for surface sterilization in most laboratories. Hyde and Soytong (2008) concluded that the isolation of endophytes is usually biased towards fast growing fungi on the isolation media. Hence, powdered plant materials

Table 3. Endophytic fungi isolated from different ages of *Ocimum sanctum* leaves on various culture media.

Leaf age maturity	Culture media (number of isolates)						Total
	PDA[1]	PHP[2]	PPE[3]	MEA[4]	MHP[5]	MPE[6]	
Young	2 ± 0.2	6 ± 0.5	3 ± 0.4	2 ± 0.3	4± 0.7	2 ± 0.2	19
Mature	3 ± 0.4	11 ± 0.8	7 ± 0.2	1 ± 0.4	7 ± 0.4	4 ± 0.4	33
Old	7 ± 0.6	17± 0.6	11 ± 0.8	4 ± 0.7	12 ± 0.9	8 ± 0.6	59
Senescent	4 ± 0.8	12 ± 0.4	6± 0.5	2 ± 0.4	7 ± 0.5	6 ± 0.5	37
Total	16	46	27	9	30	20	148

[1]Potato dextrose agar, [2]PDA + host plant powder, [3]PDA + host plant extract, [4]Malt extract agar, [5]MEA + host plant powder, [6]MEA + host plant extract.

were added to the isolation media to enhance the growth of the endophytic fungi as well as to minimize fungal contamination. This is due to the antifungal metabolites of the host which inhibits the growth of the non-endophytic fungi.

Surface imprint method and viability test conducted were used to confirm that endophytes were isolated instead of epiphytic fungi since epiphytes were killed during the immersion of leaf in 1% sodium hypochlorite (Sanchez- Marquez et al., 2007, Tong et al., 2014). Table 3 shows the isolation of endophytic fungi from various age stages on different isolation media. Six different isolation media: plain PDA, plain MEA, PDA supplemented with host plant powder (PHP), PDA supplemented with host plant water extract (PPE), MEA supplemented with host plant powder (MHP) and MEA supplemented with host plant water extract (MPE) were studied and the results showed that the isolated endophytic fungi preferred to grow on PDA and MEA supplemented with host plant powder with 47 and 31 isolates, respectively. It was followed by the PDA and MEA supplemented with host plant water extract with 28 and 20 isolates, respectively. The plain PDA and MEA managed to isolate about 16 and 9 isolates only, respectively. Table 3 also shows that a total of 148 endophytic fungi were successfully isolated from different growth stages of *O. sanctum* leaves with 19 from young (12.84%), 33 from mature (22.30%), 59 from old (39.86%) and 37 from senescent leaves (25.00%). The highest endophytic fungi were isolated from old leaf, followed by senescent, mature and young leaves.

The results obtained from this study are in agreement with Suryanarayanan and Thennarasan (2004) who demonstrated that the older leaves were more densely colonized by endophytes as compared to younger leaves, and most of endophytes obtained were fast growing fungi since they could grow after two days of incubation period. Powthong et al. (2013) defined the fast growing fungi as a group of endophytic fungi that can fill the culture plate within 5 and 7 days of cultivation. On the other hand, the slow growing endophytic fungi were the fungi that can fill the plate beyond 7 days. The results also showed that more endophytic fungi managed to be isolated on the medium supplemented with host plant powder, followed by the media supplemented with the host plant water extract. Supplementation with the host plant powder or water extract could induce the natural mutualistic interaction between endophytic fungi and their hosts, which finally enhanced the numbers of fungi isolated (Tan and Zou, 2001; Griffith et al., 2007; Zakaria et al., 2010). Furthermore, the host plant materials are believed to stimulate the biosynthesis of secondary metabolites. Besides, Tong et al. (2012) suggested that addition of host plant material in the culture media not only enhanced the growth of fungal endophytes but also able to inhibit the non-endophytic fungi.

Antimicrobial activity of endophytes isolated from different leaf ages using agar plug assay

Besides isolating the endophytes from the healthy leaves of *O. sanctum* at various leaves ages, it was also aimed to investigate the antimicrobial activity of the culturable fungi associated with it.

There were 134 isolates screened by agar plug diffusion assay to confirm if they demonstrated antimicrobial activities against 21 test microorganisms (13 bacteria, 3 yeasts and 5 fungi).

Figure 1 shows the antimicrobial activity of the endophytic fungi isolated from different leaf ages using agar plug diffusion assay. Out of the 148 isolates isolated, 134 (90.54%) were found to exhibit inhibitory activity on at least one test microorganism. The number of endophytic fungi exhibited antibacterial (17 isolates), antiyeast (6 isolates) and antifungal (4 isolates) activities. The number of isolates exhibiting antimicrobial activities increased with leaf maturity (Figure 1). According to Kruskal-Wallis H test, the number of endophytes that demonstrated antimicrobial activity is significantly different across the four leaf ages [x^2 (3, N= 60) = 15.354, p = 0.002]. Old leaves were observed to be significantly colonized by endophytes with antimicrobial activity in comparison with young leaves (U= 26.5, p = 0.000). The number of endophytes that showed activity towards bacteria was significantly higher than yeast and

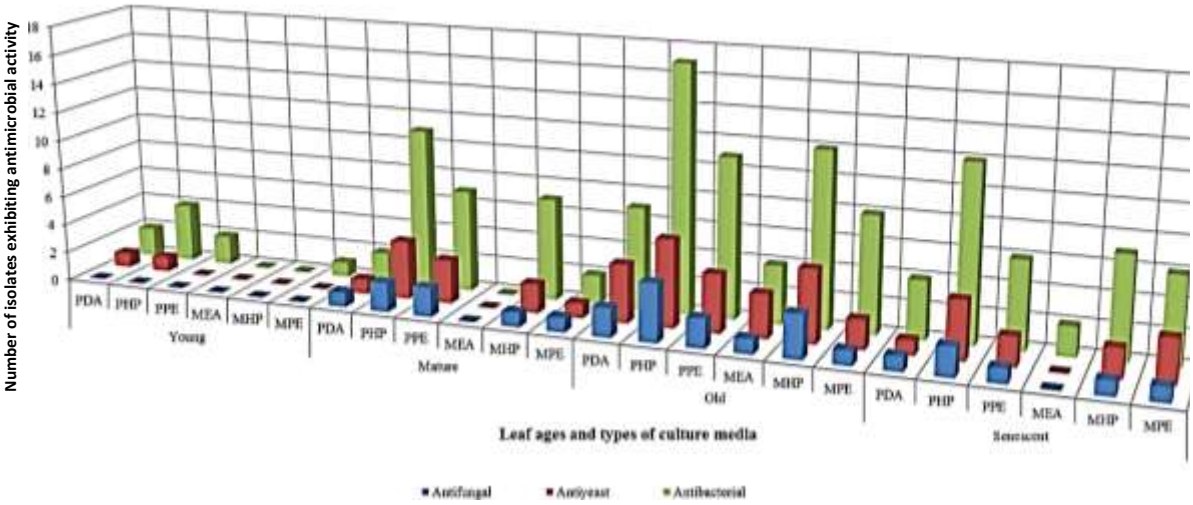

Figure 1. Antimicrobial activity of endophytes isolated from different leaf ages (young, mature, old and senescent) on various culture media [Potato Dextrose Agar (PDA), PDA supplemented with host plant powder (PHP), PDA supplemented with host plant extract (PPE), Malt Extract Agar (MEA), MEA supplemented with host plant powder (MHP) and MEA supplemented with host plant extract (MPE)].

fungi. The number of isolates that exhibited antimicrobial activity was higher in PDA agar supplemented with host plant powder as compared to PDA with host plant extract and plain PDA. The same trend also occurred for MEA where the MEA supplemented with host plant powder was colonized by more endophytic fungi with antimicrobial activity (26 isolates) as compared to MEA with host plant extract (17 isolates) and plain MEA (6 isolates). Statistically, number of isolates exhibiting antimicrobial activity grown on PDA supplement with host plant powder (U= 130, df= 1, z= -0.04, p= 0.05) showed significant difference when compared with PDA supplemented with host plant extract and plain PDA. As for MEA, the media culture supplemented with host plant powder showed significant difference as compared to media culture supplemented with host plant extract and plain media culture. The results clearly showed that the endophytic fungal isolates need compounds supplied by the host plant in order to promote their growth and enhance production of their antimicrobial compounds. Tan and Zou (2001) reported that the interaction between host and their endophytes not only benefit the host, but also the endophytes with supplement of nutrient. Moreover, the selection of media culture is also crucial in isolation of endophytic fungal with antimicrobial activity.

Several researchers have studied the relationship between the host plants and the endophytes, and they found that the addition of host plant extract can increase the growth of the endophytes as well as enhance the antimicrobial production (Firakova et al., 2007). This condition is due to a long period of relationship between the two of them that has established connection through continuum of mutualism (Jia et al., 2016) where some compounds produced by the host are essential for the endophytes. Hence, it is essential to understand such relationships and the knowledge can be well exploited and applied for the production of better and more drugs from the endophytes.

Conclusion

This study shows that *O. sanctum* harbors diverse species of fungal endophytes and some of them exhibit significant inhibitory activity on pathogenic bacteria, yeasts and fungi. A significant enhancement in antimicrobial activity of the isolates was found when the plant extracts was added to the culture medium. Further investigations on isolation of these antimicrobial compounds are crucial as an approach to search for novel natural products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors are grateful to Universiti Sains Malaysia for awarding the RUI research grant scheme (ac: 1001/PBIOLOGI/811326) to support this study.

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Vol. 12(26), pp. 623-628, 14 July, 2018
DOI: 10.5897/AJMR2018.8868
Article Number: 9694AE157877
ISSN: 1996-0808

http://www.academicjournals.org/AJM

African Journal of Microbiology Research

Full Length Research Paper

Microbiological quality of fruit juices sold in cafes and restaurants of Shewarobit town, Amhara, Ethiopia

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Received 16 April 2018; Accepted 22 May, 2018

Fresh and unpasteurized fruit juice is common in restaurants, cafeteria, hotels and juice house of Ethiopian cities. Most fruit juices contain sufficient nutrients that could support microbial growth. The current investigation was carried out to investigate the microbiological quality and processing conditions of fruit juice vended in Shewarobit town. Purposive sampling technique was employed to collect sixteen fruit juice samples comprising of eight avocado and eight papaya from five cafeterias and three restaurants of Shewarobit town from November 2017 to January 2018. A wide mouth sterile bottle was used to collect fruit juice. Collected samples were diluted and 0.1 mL inoculated on plate count agar to determine total viable count, on violet red bile agar to determine total coliform count, on mannitol salt agar to determine total *Staphylococcal* count and on Sabouraud's dextrose agar for count of yeast and molds. Questionnaires were delivered for juice vendors to obtain information on demographic characteristics and conditions for processing. There is no significant statistical difference between numbers of microbial count ($p > 0.05$). The total viable count was found in the range of 1.3×10^5 to 2.9×10^5 cfu/ mL. The total coliform count was found to be between 0.1×10^5 and 2.4×10^5 cfu/mL. *Staphylococci* count was between 0.2×10^5 and 1.7×10^5 cfu/mL. The total yeast and mould counts for all fruit juice were in the range of 1.4×10^5 to 2.7×10^5 cfu/mL. Microbiological quality of most of the fruit juices were found to be not satisfactory when compared with Gulf region standard. Therefore, regular supervision and training about safe processing and handling is very crucial for juice vendors to improve microbiological quality of fruit juice.

Key words: Fruit juice, microbial count, microbial safety.

INTRODUCTION

Fruits are important in human nutrition, supplying the necessary growth factors such as vitamins and essential minerals in daily diet to help keep a good and normal health. Juices are commonly consumed as a beverage or can be used as flavoring in foods. It is prepared by mechanically squeezing of the fruit or vegetable flesh without heating or adding solvents (Agwa et al., 2014). In many tropical countries they are common man's

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beverages and are sold at all public places and roadside shops. There are reports of food borne illness associated with the consumption of fruit juices at several places in India and elsewhere (Chumber et al., 2007; Pierre and Sivasubramani, 2015; Mahfuza et al., 2016).

Fruits and fruit juices processed under hygienic condition could play important role in enhancing consumers' health through inhibition of breast cancer, congestive heart failure (CHF), and urinary tract infection. In absence of good manufacturing practice; however, the nutritional richness of fruits and fruit juices makes the product good medium for microbial growth, vehicle of foodborne pathogens and associated complications (Mihiretie and Desta, 2015). Food borne disease has been linked with utilization of fruit juice in several areas (Kaddumukasa et al., 2017).

Microbial food borne disease harms mainly gastrointestinal tract (GI) and are transmitted via consumption of contaminated food and water with pathogens. Studies showed that fruit juice may be potential source of pathogenic bacteria particularly *E. coli* 0157:H7, *Salmonella* sp., *Shigella* sp. and *S. aureus* (Agwa et al., 2014; Abisso et al., 2018).

Fresh squeezed fruit juices are preferred by consumers due to fresh flavor attributes and currently their demand have been increased (Mahale et al., 2004). Disease causing microorganism is introduced to fruits and vegetables via punctures, cuts and splits that occur during growing and harvesting (Kulkarni et al., 2017). Colonization of raw materials and equipments, processing conditions, improper handling and unhygienic conditions contribute significantly the entrance of bacterial pathogens to juices prepared from fruits or vegetables (Nicolas et al., 2007).

Several studies on knowledge and practice of fruit juice vendors regarding food safety was investigated in several countries indicating the gap between knowledge on food hygiene and handling practices (Babiye, 2017; Senbeta and Beyene, 2017). The urban residents of Ethiopia purchase canned or bottled forms of fruit juice available in supermarkets. In addition fruit juice vending houses serving different types of fruit juices in fresh forms are increasing. However, the scientific information on microbiological quality and safety of fruit juice prepared and consumed in Ethiopia is scanty. Therefore the objective of this study was to evaluate the microbiological quality of fruit juices and to assess the juice vendors knowledge on processing conditions at Shewarobit town, North showa zone, Ethiopia.

MATERIALS AND METHODS

Study site

The study was conducted at Shewa Robit town located at 225 Km North east of Addis Ababa at a latitude of 9°59'60.00"N and Longitude of 39°54'0.00" E with an elevation of 1280 m above sea level. According to the 2007 Census Survey Report of the Ethiopian Central Statistical Agency, the town has a total population of 17,575.

Sample collection

Eight Avocado and eight Papaya fruit juice were collected using purposive sampling technique from service delivering five cafeterias and three restaurants in Shewarobit town from November 2017 to January 2018. All samples of fruit juice were collected on a voluntary basis from restaurants and cafes of Shewarobit town. A wide mouth sterile (250 mL) bottle was used for collection of juice samples and transported to laboratory in an ice-box, and processed immediately. Questionnaires were used to obtain information on the socio-demographic characteristics of juice vendors and hygienic quality. Questionnaires were prepared in English and translated into local language Amharic.

Microbial analysis

One millilitre (1 mL) of each liquid juice sample was taken and transferred into sterilized cotton plugged test tubes containing 9 mL of sterile peptone water, mixed thoroughly by shaking many times for 10-fold serial dilution aseptically under laminar flow. Appropriate dilutions of 0.1 mL of juice sample was inoculated on surfaces of respective media for microbial count using spread plate technique in duplicates. All culture media used for microbial analysis were from Himedia Laboratories Ltd, Mumbai, India. Plate Count Agar used for cultivation of total viable bacterial count after incubation of 32°C for 48 h. Violet Red Bile Agar (VRBA) was employed for enumeration of coliforms after incubation at 32°C for 48 h. *Staphylococci* were cultured on Mannitol Salt Agar (MSA) and counted after incubation at 32°C for 48 h. Yeast and molds count was conducted after incubation of 3 to 5 days on Sabouraud's dextrose agar (SDA) at 25 to 28°C. 0.1 g of chloramphenicol was added to the media to inhibit bacterial growth (Titarmare et al., 2009).

The countable colonies were converted into weighted mean colony forming units per millilitre (cfu/mL) using the formula; $N=\Sigma C/(n1+0.1n2)\ d$ where; N=the number of bacteria counted, C= sum of colony counted in two successful dilutions, n1=the number of dishes retained in the first dilution, n2= the number of dishes retained in the second dilution and d= dilution factor corresponding to the first dilution. Two consecutive plates with 15 to 30 colonies were considered for record (Simforian et al., 2015).

Statistical analysis

Questionnaires were delivered to respondents to determine the factors related to microbiological quality and safety of fruit juice. The collected questionnaires from respondents were analysed and presented using descriptive statistics. The microbial counts of fruit juice grown on culture medium (total coliform count, *Staphylococci* count, total viable count and yeast-mold count) were analyzed using one-way ANOVA. *P*-value less than 0.05 were considered statistically significant.

RESULTS

Demographic characteristics of participants

All of 16(100%) of the fruit juice makers who participated in this study were females and 2 (12.5%) of them were older than 35 years of age, 10(62.5%) of the participants

Table 1. Fruit juice processing conditions of vendors in Shewarobit town (N=16).

Variable	Number of respondents	Percent (%)
Type of vender		
Restaurant	4	25
Cafe	12	75
Source of fruits		
Open market	14	87.5
From producer	12	12.5
Nature of fruits		
Ripened	14	87.5
Over-ripened	12	12.5
Storage site of fruits before processing		
Shelf	6	37.5
Basket	6	37.5
Refrigerator	4	25
No special storage	-	-
Water source for juice preparation		
Well	-	-
Tap	16	100
Spring	-	-
Other specify	-	-
Food hygiene and safety training		
Yes	-	-
No	16	100
Microbes can contaminate fruit juice		
Yes	13	81.25
No	3	18.75
Hand washing habit before preparing fruit juice		
Yes	12	75
No	4	25
Washing habit of fruits before making juices		
Yes	4	25
No	12	75

were 15-35 years old and 4(25%) of the participants were less than 15 years old. The education status of juice makers result revealed that 5 (31.25%) had completed primary education; 8 (50%) of juice makers had acquired higher than primary education while only 3(18.75%) of the participants had non-formal education.

Hygienic safety of fruit juices

The source of fruits used for the processing of juices was primarily from the open market (87.5%) while some juice makers (12.5%) got their fruits directly from producers who were their routine suppliers. Fruit juice makers made use of both ripened and over-ripened fruits but with preference to ripened fruits (87.5%) of the cases. Fruit juice makers use shelves (37.5%), baskets (37.5%), and refrigerators (25%) for storing fruits before processing. Moreover, all of the fruit juice makers (100%) used tap water for washing fruits before preparation of fruit juices (Table 1).

Microbiological enumeration of fruit juices

The microbiological load of avocado and papaya juice

Table 2. Microbiological profiling of (Avocado and Papaya) juices sold in Shewarobit café and restaurants (n=16).

Sample code	Total viable count (cfu/mL)	Total coliform (cfu/mL)	Staphylococci (cfu/mL)	Yeasts and molds (cfu/mL)
AJ1	2.2×10^5	2.1×10^5	1.1×10^5	2.3×10^5
AJ2	2.1×10^5	1.8×10^5	1.5×10^5	2.3×10^5
AJ3	1.6×10^5	1.1×10^5	0.7×10^5	1.8×10^5
AJ4	2.8×10^5	2.0×10^5	1.2×10^5	2.4×10^5
AJ5	2.3×10^5	2.2×10^5	1.3×10^5	2.6×10^5
AJ6	2.7×10^5	2.3×10^5	1.7×10^5	2.5×10^5
AJ7	2.9×10^5	1.5×10^5	1.2×10^5	1.9×10^5
AJ8	2.5×10^5	2.4×10^5	1.4×10^5	2.7×10^5
PJ1	1.9×10^5	0.5×10^5	0.2×10^5	2.0×10^5
PJ2	1.3×10^5	0.1×10^5	0.6×10^5	1.4×10^5
PJ3	1.4×10^5	1.1×10^5	0.7×10^5	1.7×10^5
PJ4	1.9×10^5	0.1×10^5	0.4×10^5	2.5×10^5
PJ5	2.0×10^5	1.6×10^5	0.6×10^5	2.6×10^5
PJ6	1.7×10^5	1.4×10^5	1.6×10^5	2.3×10^5
PJ7	1.8×10^5	1.7×10^5	0.8×10^5	2.2×10^5
PJ8	2.1×10^5	1.3×10^5	1.5×10^5	1.9×10^5

AJ = Avocado juice; PJ = papaya juice.

analyzed for total bacterial viable count, coliform count, *Staphylococci* count, yeast and mold count is depicted in Table 2. Avocado juices were found to have microbial count in the range of 0.7×10^5 (AJ 3) to 2.9×10^5 (AJ 7) cfu/ mL. On the other hand, papaya juices were found to have microbial count in the range of 0.1×10^5 (PJ2 &4) to 2.6×10^5 (PJ5) cfu/mL. The total yeast and mould counts for all fruit juice were in the range of 1.4×10^5 to 2.7×10^5 cfu/mL. Separately, the total viable count lies in the range of 1.6×10^5 to 2.9×10^5 and 1.3×10^5 - 2.1×10^5 cfu/ mL for avocado and papaya juice, respectively.

The total coliform count for Avocado juice was found to be between 1.1×10^5 and 2.4×10^5 cfu/mL while Papaya juice coliform count was between 0.1×10^5 and 1.7×10^5 cfu/mL. *Staphylococci* count was between 0.7×10^5 and 1.7×10^5 cfu/mL for Avocado juice and 0.2×10^5 to 1.6×10^5 cfu/mL for papaya juice. Mold and yeast count for avocado juice was in the range of 1.8×10^5 and 2.7 and 10^5 cfu/mL and 1.4×10^5 to 2.6×10^5 cfu/mL for papaya juice.

Analysis of one way ANOVA showed that the absence of significant statistical difference between number of microbial count (Total coliform count, Staphylococci count, Total viable count and Yeast-mold count) ($P > 0.05$).

DISCUSSION

Laboratory examined samples of avocado and papaya juice were found to be contaminated with different groups of microorganism. All samples of Avocado and Papaya of fruit juices were found to have microbial count in the range of 0.1×10^5 to 2.9×10^5 cfu/mL. Ketema et al. (2008) reported in Jimma town that the range of microbial counts recorded in the fruit juice analyzed were from 3.1×10^7 to 6.2×10^3cfu/mL.

The total viable bacterial count of all collected fruit juice in this investigation varies from 1.3×10^5 to 2.9×10^5 cfu/mL; this bacterial count was relatively lower than that of Singh et al. (2015) who reported that all samples of street vended fruit juices were found to have bacterial count in the range of 10^5 to 10^8 cfu/ mL. In other studies conducted by Ankur et al. (2009), the quantitative analysis of samples for total viable count (TVC) revealed that the range for TVC were between 1.0×10^4 to 4.0×10^6 cfu/mL.

According to study conducted in Kolkata city India, juice samples collected from most populated market places of and summarized account of the results obtained for the microbiological analysis of the juices; total viable counts were high ranging from 265-700 $\times 10^4$ cfu/1000 mL (Mahuya et al., 2011).

However, the recommended specifications for fruit juices such as Avocado, Mango, Pineapple and Papaya available for public consumption in Gulf region suggest that the maximum count for Total viable count, Coliform count and Yeast-Mold count are 1×10^4, 1×10^2 and 1×10^3 cfu/mL, respectively. According to the specification of Gulf standards, the colony counts of all microbial groups (total coliform count, *Staphylococci* count, total viable count and Yeast-mold count) in this study exceeded the standard by considerable margin. The higher counts, however, may not necessarily pose hazard to the health of consumers provided that probably there are no potential pathogenic strains such as strains of *E. coli* and

Salmonella species within the fruit juices to be consumed (Babiye, 2017).

The total coliform count for investigated fruit juice was found to be between 0.1×10^5 and 2.4×10^5 cfu/mL. According to study conducted in Dhaka, Bangladesh, out of 114 freshly prepared fruit juices samples collected, 113 samples (99%) were positive for coliform and *E. coli* (Shakir et al., 2009). Coliforms include both the presence of faecal (0.05- 45×10^4 cfu/1000 mL) and non-faecal ($0.15\text{-}76\times10^4$ cfu/1000 mL) (Mahuya et al., 2011). The result was greater than Gulf region standard with limited colony count of coliform not more than 100 cfu/mL in fruit juice. It is contended that contamination is mainly due to poor quality of water used for dilution as well as prevailing unhygienic conditions related to washing of utensils and maintenance of the premises. Presence of coliforms in high numbers (100 cfu/mL) was health hazard causing spoilage of fruit juices and food borne diseases.

The finding of the current result revealed that the *Staphylococci* count was between 0.2×10^5 and 1.7×10^5 cfu/mL for all fruit juice. The presence of *Staphylococci* in high numbers (10^3 cfu/mL) is a health hazard as they cause spoilage of fruit juices and food borne diseases (Gulf standards, 2000). The existence of *S. aureus* in fruit juice can contribute to contamination through handling and processing conditions.

The total yeast and mould counts for all fruit juice analyzed in the current investigation were in the range of 1.4×10^5 to 2.7×10^5 cfu/mL. According to the study conducted on the microbiological quality of freshly squeezed or freshly prepared fruit juices sold by local market vendors in Dhaka city; the total fungal counts were in the range of 1.0×10^1 to 8.05×10^4 cfu/mL (Shakir et al., 2009). Fungal fruit contamination may occur during the growing season, harvesting, handling, transport and post-harvest storage and marketing conditions (Al-Hindi et al., 2011). Fruits contain high levels of sugars and nutrient elements and their low pH values make them particularly desirable to fungal growth which in turn may result in their decay (Singh and Sharma, 2007). But the result of this study is more than the standard set by Gulf Region Mould and yeast count (1×10^3 cfu/mL).

Conclusion

From the present finding, the microbiological quality of most of the vendor fruit juices was found not to be satisfactory for consumption as compared to Gulf region standard. The total bacterial count, yeast and mould count, coliform count and *Staphylococci* count of investigated fruit juice was also alarming. All samples of Avocado and Papaya of fruit juices were found to have microbial count in the range of 0.1×10^5 to 2.9×10^5 cfu/mL. All of the study participants (100%) responded to the absence of training in food hygiene and safety. Therefore, training about safe processing and handling is very crucial for juice vendors to improve microbiological quality of fruit juice.

CONFLICT OF INTERESTS

The author declares no conflict of interest.

ACKNOWLEDGEMENTS

This research paper would not have been possible without the help of many people who supported the authors during their study period. The authors would like to express their heartfelt thanks and deep appreciation to Debre Berhan University, research directorate for financial support to conduct the study.

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