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Full Length Research Paper

Using light emitting diodes at 450 nm for *in vitro* treatment of water intended for human consumption

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This study aimed to evaluate the *in vitro* antimicrobial effect of exposing raw water intended for human consumption to light (λ= 450 nm) and to investigate the correlation between the results obtained and physical and chemical parameters. Fifteen (15) samples of raw water were collected from households in a rural area of *Santo Antônio de Jesus* – Bahia (Brazil), from November to December 2016. A 100 mL aliquot of each sample was exposed to a lighting system consisting of two high intensity light emitting diodes, with a wavelength of 450 nm and luminous flux of 200 lumens per 10 h. Quantifications of heterotrophic bacteria, total coliforms and temperature started at time zero and were done every two hours until the end of exposure to light. Bacteriological analysis was repeated after 72 h of being exposed to light. pH, dissolved oxygen and salinity analyses were performed before each experiment. After a 10h illumination at 450 nm light emitting diodes (LEDs), the dosage of light received by the water samples was 581.8 J/cm^2. There was a significant reduction in the two bacteriological parameters analyzed after treatment (p = 0.000). There was an average decrease in heterotrophic bacteria counts from 3.44 to 1.86 log CFU/mL and total coliforms from 2.45 to 1.02 log CFU/mL. Mean reductions of heterotrophic bacteria were 97.01% and total coliforms were 95.61%. After 72 h, both counts increased; there was significant growth between heterotrophic bacteria (p = 0.000), but there was no significant growth for total coliforms (p = 0.058). pH (p = -0.981, p = 0.000), dissolved oxygen (ρ = -0.529, p = 0.043) and temperature (ρ = 0.521, p = 0.047) were related to the percentage reduction of heterotrophic bacteria. The method is shown to be effective in disinfecting raw water *in vitro* under different physical and chemical conditions.

Key words: Blue light emitting diode (LED), indicator microorganisms, potability standards, contaminated water.

INTRODUCTION

Light has been used for decades as a bacterial inactivation tool in several areas. Sunlight is used for

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water disinfection because it has combined antimicrobial action of ultraviolet A (UVA), is responsible for the alteration of microorganisms' DNA and infrared radiation that causes water temperature to increase. However, with low light intensity, the process is not effective (Boyle et al., 2008; Mcguigan et al., 2012).

Artificially produced ultraviolet (UV) radiation is also used for water treatment. Ultraviolet band C (UVC - 254 nm) is used as germicide. However, in sublethal doses, microorganisms can recover their metabolic activity, in addition to requiring a physical barrier to protect the operator due to its carcinogenic potential (Wisbeck et al., 2011; Di-Bernardo et al., 2017; Mbonimpa et al., 2018).

However, recently, with the introduction of new technologies, researches evaluating the antimicrobial effect of light emitting diode (LED) in visible light spectrum and ultraviolet light spectrum have emerged (Maclean et al., 2014). A LED is a compact electronic device that emits light within a monochromatic wavelength spectrum, when electric current passes through it. The main advantage LED has over traditional devices is its high durablility, with a life expectancy of approximately 30,000 h, and consumes low energy when producing a high luminous flux. In the visible light spectrum, due to its safety, it does not require additional protection of its operator (Ghate et al., 2013; Yeh et al., 2015).

Recent studies using this apparatus demonstrate that after the exposure of bacteria to violet or blue light at a wavelength between 405-520 nm, they are inactivated. The device can be potentially applied in food and clinical microbiology, since it has the ability to inactivate pathogenic bacteria, such as S*taphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli* O157: H7, *Salmonella* spp., *Staphylococcus epidermidis*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii*, *Proteus vulgaris*, *Klebsiella pneumoniae* (Enwemeka et al., 2008; Maclean et al., 2009; Ghate et al., 2013; Kumar et al., 2015). Its cytotoxic action is widely documented and is characterized from the formation of reactive oxygen species (Guffey and Wilborn, 2006; Luksiene and Zukauskas, 2009). Bacteria, such as *Salmonella* spp., *S. aureus*, *Escherichia coli*, and *Bacillus cereus* are amongst the predominant species identified as etiological agents responsible for outbreaks of foodborne diseases in Brazil, from 2007 to 2016. These microorganisms account for 77.8% of all outbreaks in that time interval in which the etiological agent was identified (Brazil, 2016).

Considering the possibility of bacterial inactivation after exposure to visible light at monochromatic wavelengths, the efficiency of this system against pathogenic bacteria causing foodborne diseases and the lack of researches on the use of this device for treatment of water meant for human consumption, this study aims to evaluate the *in vitro* antimicrobial effect of exposing raw water intended for human consumption to light at 450 nm and also to investigate the relationship of the results with physical and chemical parameters.

MATERIALS AND METHODS

Samples

Raw water samples were collected from 15 households located in a rural area of *Santo Antônio de Jesus – Bahia* (Brazil), from November to December 2016. 500 mL of water was collected and stored in polyethylene bottles used for the first time. Samples were labeled and stored in thermal boxes, and kept at refrigeration temperature (+2 to + 8°C). They were transported to the Laboratory of Microbiology and Parasitology of the Center of Food and Nutrition Security (SANUTRI) of Health Science Center (CCS), Federal University of Recôncavo of Bahia (UFRB), where they were kept in the refrigerator for 12 h.

Characterization of the light emitting diodes system

The lighting system consists of two high-intensity LEDs, each of 10 W, with a wavelength of 450 nm (blue) and luminous flux of 200 lumens. The LED was put on a heat dissipating plate in order to minimize heat transfer from the LED to the samples. It involved using an acrylic plate to isolate the samples from the environment. The entire system was overlaid with an autoclaved glass vat. This way, the LED was positioned directly above each sample (Ghate et al., 2013; Kumar et al., 2015) (Figure 1).

Experimental arrangement

A 100 mL aliquot of the refrigerated sample was transferred to the glass vat. The LED system was put on the vat, switched on and transferred to the refrigerator. It was kept under this condition for 10 h. Bacteriological analysis started at time zero and was performed every two h for 10 h. After 10 h, the samples were stored in polyethylene bottles used for the first time under the same temperature conditions, but they were protected from light. After 72 h, a new analysis was performed. Consequently, there were seven analyses done for each sample. A control sample was maintained under the same refrigeration conditions, but it was not exposed to the LED light. Bacteriological analysis in the control sample was performed at the beginning of the experiment and after 72 h (Ghate et al., 2013; Kumar et al., 2015). The dosage of light received by each water sample was calculated using the equation:

$$E = P \times T$$

Where, E = Dose in J/cm^2; P = Irradiance in W/cm^2; and t = time in seconds (Ghate et al., 2013).

Two indicator microorganisms present in Ordinance MS 2914/2011 (Brazil, 2011) were used for bacteriological analysis. Quantification of heterotrophic bacteria was used to identify flaws in water disinfection and quantification of total coliforms, to evaluate the efficiency of the treatment (Brazil, 2011). Heterotrophic bacteria and total coliforms populations were estimated by the methods of Petrifilm Aqua Heterotrophic Count Plate (AQHC, 3M Company™) and Petrifilm Aqua Coliform Count Plate (AQCC, 3M Company™), respectively. 1 mL of the sample was diluted in 9 mL of 0.9% NaCl solution. 1 mL of the dilution was inoculated into each Petrifilm plate. After complete gel solidification, plates were incubated in a bacteriological oven at 36 ± 1°C for 44 ± 4 h and 36 ± 1°C for 24 ± 2 h, respectively. Results were expressed as log CFU / mL (APHA, 2012). pH, dissolved oxygen and salinity analyses were performed

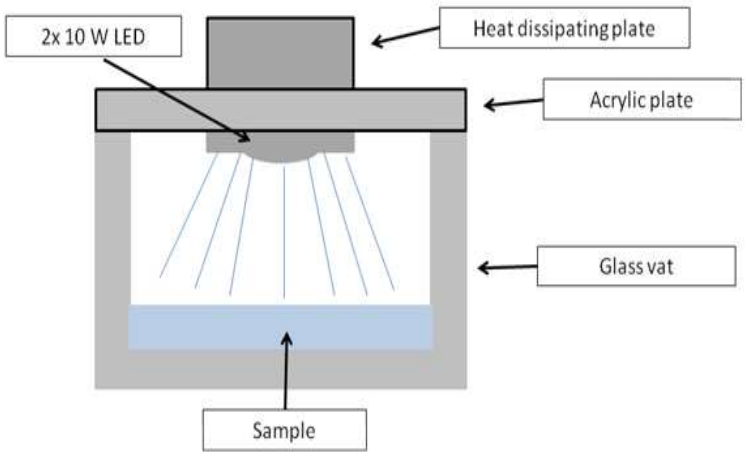
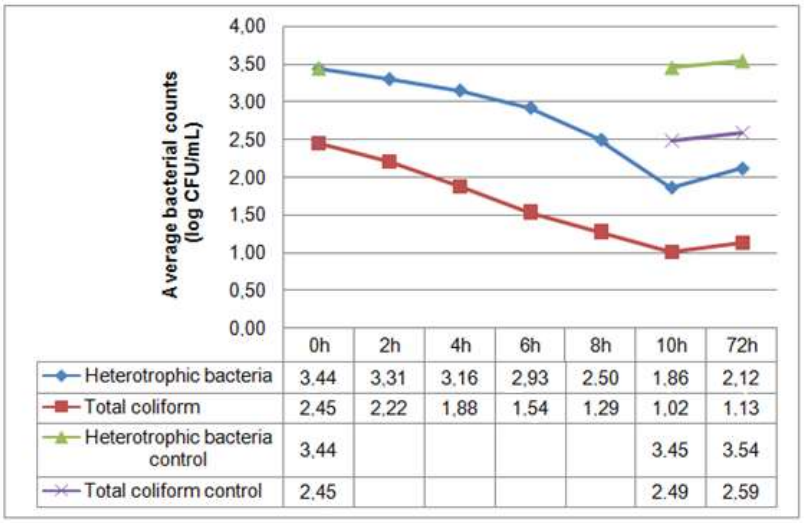


Figure 1. Diagram of the experimental LED lighting system.



	0h	2h	4h	6h	8h	10h	72h
Heterotrophic bacteria	3,44	3,31	3,16	2,93	2,50	1,86	2,12
Total coliform	2,45	2,22	1,88	1,54	1,29	1,02	1,13
Heterotrophic bacteria control	3,44					3,45	3,54
Total coliform control	2,45					2,49	2,59

Figure 2. Bacterial inactivation after samples exposure to 450 nm light.

before each experiment with a AK88 multiparameter meter (AKSO®), using an approximate aliquot of 100 mL of water. Temperature was checked with an infrared digital thermometer MT-350 (Minipa®) every two hours. Color and turbidity analysis was performed with a spectrophotometer SP22 (Biospectro), using wavelengths of 455 nm and 860 nm, respectively (APHA, 2012).

Statistical analysis

Data were processed and analyzed using Microsoft Office Excel version 2007 (Microsoft Corporation™) and Statistical Package for the Social Sciences (SPSS) version 23 (International Business Machines™). The data normality test (Shapiro-Wilk) was performed with all quantitative variables. Descriptive and analytical statistics, such as mean, median, maximum and minimum, logarithmic and percentage reduction, Spearman's correlation coefficient, paired *t* test and analysis of variance (ANOVA) were performed. The level of significance was 5% ($p < 0.05$).

RESULTS

Figure 2 shows the means of the bacterial populations of the fifteen samples analyzed within 10 h of exposure to light as well as bacterial growth after 72 h. There was a decrease in heterotrophic bacteria and total coliforms counts during their exposure to light (3.44 to 1.86 log CFU/mL) and total coliforms (2.45 to 1.02 log CFU/mL). After 72 h, both counts increased, with significant growth of heterotrophic bacteria ($p = 0.000$), but there was no significant growth for total coliforms ($p = 0.058$). Even after 72 h, samples exposed to light, when compared to control samples, obtained values 26 times lower for heterotrophic bacteria and 30 times lower for total coliforms. After a 10 h illumination by the 450 nm LEDs, the dosage received by the water samples was 581.8

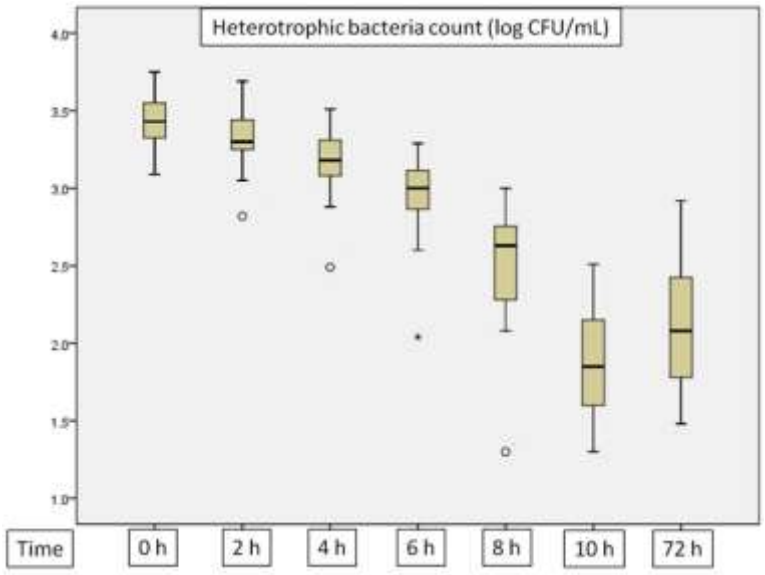


Figure 3. Bacteriological quantification during 10 h of experiment and after 72 h.

J/cm^2.

Quantifications of heterotrophic bacteria before their exposure to light reached a maximum value of 3.75 log CFU/mL, median of 3.63 log CFU/mL and a minimum value of 3.09 log CFU/mL. All the values were above the ones recommended by the Brazilian Legislation (Brazil, 2011), 11.4 times higher than the recommended ones (limit of 2.7 log CFU/mL). After 10 h of the bacteria exposure to light, the maximum value found was of 2.51 log CFU/mL and the minimum was 1.30 log CFU/mL, meaning all samples were within the legislation standards. Within 8 h of their exposure, most samples were already within this legislation standard (73.3%). Regarding total coliform counts, a maximum value of 3.08 log CFU/mL, median of 2.48 log CFU/mL and a minimum value of 1.90 log CFU/mL were found before the bacteria exposure to light. After 10 h of their exposure, maximum value was reduced to 1.48 log CFU/mL, median and minimum values to <1 log CFU/mL. In eight samples there were no counts of total coliform after 10 h of the bacteria exposure to light, nor growth after 72 h (Figure 3).

There was a significant reduction in the two bacteriological parameters analyzed after treatment ($p = 0.000$). Reductions in bacterial counts reached 98.98% for total coliforms and 98.96% for heterotrophic bacteria. The lowest reductions found were 88.75% in total coliform analysis in sample 12 and 93.57% in the heterotrophic bacteria analysis in sample 8. Mean reduction of the heterotrophic bacteria was 97.01% and of total coliforms, 95.61%. During pre and post treatment periods, there is strong positive and significant correlation in the heterotrophic bacteria counts ($\rho = 0.912$; $p = 0.000$) and a moderate positive and significant correlation in total coliform counts ($\rho = 0.581$; $p = 0.023$) (Table 1).

Results of Spearman's correlation coefficient between percentage reductions of bacteriological parameters and physical and chemical analysis showed a strong negative and significant correlation between heterotrophic bacteria percentage reduction and pH ($p = 0.000$), moderate positive and significant correlation between heterotrophic bacteria percentage reduction and dissolved oxygen ($p = 0.043$) and moderate negative and significant correlation between the percentage reduction of heterotrophic bacteria and temperature ($p = 0.047$) (Table 2). pH values reached a maximum of 6.31; median, 5.0 and a minimum, 4.15. Dissolved oxygen had a maximum value of 9.50 mg/L; median, 5.70 mg/dL and minimum, 4.0 mg/dL. Temperature reached a maximum value of 8°C; median, 7°C and minimum, 6°C. There was an increase of 0.5-1.0°C in each experiment, but temperature did not exceed 8°C. Salinity had the lowest variation, with a maximum value of 0.06 ppm, median of 0.03 and 0.02 ppm. Color and turbidity obtained zero values in all samples (Figure 4).

DISCUSSION

Results demonstrate the antimicrobial effect of high intensity light with a 450 nm wavelength under refrigeration temperature on tested indicator micro-organisms. Reductions were significant during the experiment, indicating the efficiency of the process in small scale. The bacterial inactivation process has been shown to be dose dependent, because reductions result from longer exposure time. Inactivation curves and percentage reductions were similar and sometimes better

Table 1. Reductions of heterotrophic bacteria and total coliforms counts after 10 h exposure to light at 450 nm.

Sample	Heterotrophic bacteria		Total coliforms	
	Logarithmic reduction (log CFU/mL)	Percent reduction (%)	Logarithmic reduction (log CFU/mL)	Percent reduction (%)
1	1.45	96.48	1.99	98.98
2	1.66	97.83	1.41	96.09
3	1.53	97.01	1.35	95.50
4	1.49	96.73	1.51	96.88
5	1.43	96.27	1.70	98.00
6	1.27	94.58	1.22	94.00
7	1.98	98.96	1.35	95.50
8	1.19	93.57	1.60	97.50
9	1.75	98.21	1.09	91.82
10	1.27	94.59	1.74	98.16
11	1.70	98.00	1.16	93.08
12	1.79	98.36	0.95	88.75
13	1.84	98.55	1.52	97.00
14	1.71	98.04	1.33	95.35
15	1.68	97.93	1.61	97.57

Table 2. Spearman's correlation coefficient between percentage reductions and physical and chemical analysis.

Parameter	Percentage reduction of heterotrophic bacteria	Percentage reduction of total coliforms
pH	-0.981[b]	0.462
Dissolved oxygen	0.529[a]	-0.041
Temperature	0.521[a]	0.055
Salinity	-0.104	-0.588

[a]The correlation is significant at the 0.05 level; b - The correlation is significant at the 0.01 level.

compared to previous studies done with pathogenic bacteria like *S. aureus, S. pyogenes, Bacillus cereus, Listeria monocytogenes, Escherichia coli* O157:H7 (Guffey and Wilborn, 2006; Enwemeka et al., 2008; Maclean et al., 2009; Ghate et al., 2013; Kumar et al., 2015).

Microorganisms are sensitive to environmental changes. There is an inversely proportional relationship between pH and percentage reduction of heterotrophic bacteria, and, the more the pH moves away from neutrality, the greater the percentage of bacterial reduction. Optimum pH ranges from 6.5 to 7.5 for most bacteria, thus lower values can inhibit or delay bacterial multiplication. A few species of bacteria, such as *E. coli* have survival mechanisms at more acidic pH in short periods. However, bacterial growth may fall by up to five times at low pH (Cotter and Hill, 2003; Rousk et al., 2009).

The process of bacterial inactivation using visible light is oxygen dependent, according to Feuerstein et al. (2005). Photodynamic inactivation involves excitation of photosensitizing molecules, such as endogenous porphyrins. Excitation of porphyrin leads to energy transfer, which, on the other hand, generates reactive oxygen species, especially singlet oxygen. These reactive oxygen species oxidize constituents of the cell membrane, such as unsaturated fatty acids, proteins, in addition to DNA, promoting a cytotoxic and bactericidal effect (Hamblin and Hasan, 2004; Guffey and Wilborn, 2006; Luksiene and Zukauskas, 2009).

It is possible that species less tolerant to oxygen are more susceptible to these cytotoxic effects, due to the lower presence of oxidative regulatory mechanisms compared to aerobic species (Luksiene and Zukauskas, 2009; Murdoch et al., 2010; 2012). Even at low temperatures, some studies have shown that bacterial damage can rise with higher temperatures; as bacteria increase their metabolic rates in these situations, their metabolic load and cytotoxic reactions that aid their inactivation also increase (Song et al., 2011; López-Velasco et al., 2012). However, the increased susceptibility of bacteria observed in this and other studies may be related to the increase of unsaturated fatty acids in the cell membrane. These unsaturated fatty

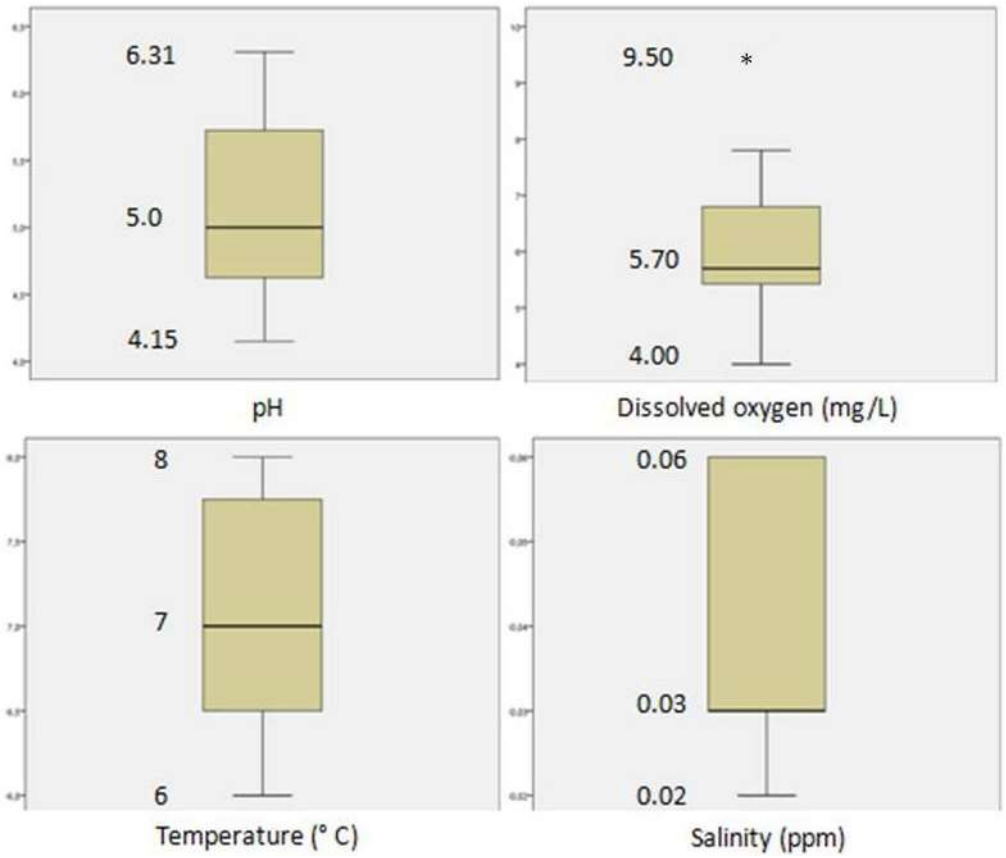


Figure 4. Physical and chemical evaluation of samples.

acids have a greater tendency to be oxidized compared to saturated fatty acids, therefore a greater damage will occur to this important cellular constituent. The optimal temperature for bacterial inactivation in this light spectrum is close to 10°C (Ghate et al., 2013; Kumar et al., 2015).

Bacterial growth after 72 h can be explained by the presence of particulate matter and colonies in the medium with bacterial aggregation; this negatively affects the water disinfection process with light exposure, as they lead to the formation of areas with no direct exposure to light and consequently oxidative stress will be affected. Thus, raw water needs to be filtered (Bohrerova and Linden, 2006; Cantwell and Hofmann, 2008). Another possibility would be the susceptibility of species to inactivation at this wavelength. For example, *Pseudomonas aeruginosa* requires higher doses compared to S. *aureus* (Guffey and Wilborn, 2006). *Salmonella entericae Enterococcus faecalis* are two studied bacteria with greater resistance to inactivation by exposure to visible light, requiring long periods of exposure. The reasons for these different susceptibilities are still indeterminate, but analysis indicates that gram-positive bacteria tend to be more susceptible to inactivation than gram-negative (Maclean et al., 2009; 2014; Murdoch et al., 2012). Therefore, there will be cells that cannot be injured and can multiply in the time interval. Even after 72 h, there was no growth of total coliforms in 53.3% samples and 86.7% were within the recommended standard for heterotrophic bacteria (Brazil, 2011).

Compared to UV light, the germicidal efficacy of blue light is lower, since its lethal dose is much lower, thus it has a slower inactivation. However, the fact that this wavelength lies within the visible light range and does not require the necessary protections for UV light, it can be continuously for treatment of water, food as well as surfaces. It can be safely used in the presence of people in the same enclosure, it is easy to operate, and it has high penetration power in water, plastics and glass (Maclean et al., 2014).

In summary, exposure to light at 450 nm wavelength led to mean reductions in bacterial counts above 95%. All samples were within the standards recommended by the Brazilian Legislation for heterotrophic bacteria and 53.3% of the samples were within the recommended standards for total coliforms. pH, temperature and dissolved oxygen were directly related to the percentage reduction of heterotrophic bacteria.

This is an unpublished study involving raw water meant for human consumption, addressing the correlation between physical and chemical parameters and indicator

microorganisms. No other study so far has worked with these parameters or indicator microorganisms; they have used only pathogenic bacteria and experimental inoculations in Petri dish with growth medium or saline solution. Because of its uniqueness, this study presents itself as an initial effort to find new methods for water disinfection. It proves to be a safe, effective method against indicator microorganisms, allowing continuous use without the need for special protection and it is easy to use. In the same way, it demonstrates the necessity of expanding subsequent studies, seeking to perfect the system to leave experimental work i*n vitro* for *in loco* evaluations.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Quality of water for human consumption in a rural area community from Brazil

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This work aimed at evaluating the bacteriological, parasitological, physical and chemical quality of water intended for human consumption in a community in a rural area of Recôncavo of Bahia (Brazil) and the factors related to a possible contamination. Samples were collected at two different times: at rainy season (August to September, 2015) and dry season (April 2016). The present work evaluated the presence of total coliforms and *Escherichia coli*; quantified heterotrophic bacteria; performed parasitological techniques of direct examination and modified Faust; analyzed pH, temperature, dissolved oxygen, apparent color, turbidity and salinity; and applied a questionnaire regarding the water source and its storage. Out of the 53 households, 67.9% were in disagreement with bacteriological standards of potability. 5.7% in disagreement with parasitological standards, 92.5% in disagreement with physical and chemical standards and all samples were in disagreement with the parameters allowed and recommended by the Brazilian legislation. The water source, presence of a household reservoir, sewage destination and reservoir cleaning time were significantly related to the bacteriological results. The consumption of this water poses a risk to the population's health and it could provoke outbreaks of food borne diseases. Effective water treatment and surveillance measurements should be taken in order to minimize risks to human health.

Key words: Potable standards, groundwater, indicator microorganisms, contaminated water.

INTRODUCTION

Access to treated water is a basic human right (WHO, 2015). All water intended for human consumption provided from alternative supply sources, regardless of the way of access, is subject to water quality monitoring. Therefore, potable water intended for ingestion, food preparation and personal hygiene, regardless of its source, must meet established drinking standards (Brazil, 2011; Benedict et al., 2017).

Water could suffer contamination at the source, during its distribution, as well as in household reservoirs. About 10% of the world's population does not have access to drinking water and 35% have no access to basic sanitation (WHO, 2015). Brazil possesses the world's largest fresh water reservoirs, however, due to its

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territorial extension, distribution is heterogeneous. Due to the absence of effluent treatment systems, many times water is contaminated from the water table (Brazil, 2005; Rebouças et al., 2015).

Foodborne diseases represent an important public health issue in Brazil and in the world, being associated with multiple etiological agents, such as bacteria, viruses, helminths and protozoa, which invade human body through contaminated water and/or food. Despite the high occurrence rates, most cases of foodborne diseases are not reported (Brazil, 2010; Martins et al., 2015; Neves et al., 2016). Approximately 80% of all diseases in developing countries are caused by non-potable water and poor sanitation (Brazil, 2010, 2016; WHO, 2015).

Considering the shortage of studies correlating microbiological and parasitological, physical and chemical results as well as the shortness of researches regarding possible contamination factors and the relevance of studies to subsidize actions aiming to improve health of the peasant population, this study aimed to evaluate the bacterial, parasitological, physical and chemical quality of water intended to human consumption in a rural area community of Recôncavo of Bahia (Brazil), and also to investigate factors related to a possible contamination.

MATERIALS AND METHODS

Sample collection

This research is characterized as a descriptive and analytical cross-sectional study. Water samples were collected in 53 households of a rural area of Santo Antônio de Jesus-Bahia (Brazil), from August to September 2015 (end of rainy season) and repeated in 34 households in April 2016 (end of dry season), totaling 87 samples. The number of households varied according to availability of residents. Samples were collected after the reservoir. In the absence of faucets, samples were collected directly from the reservoir. Approximately, 1.5 L of water were collected. Samples obtained were stored in first use polyethylene flasks (VidroPET-Goiania, Goias, Brazil), labeled and packed in thermal boxes with recyclable ice packs, kept in the refrigeration temperature (+2 to +8°C), and analyzed within 6 h of collection at the Laboratory of Microbiology and Parasitology of Food and Nutrition Security Center (SANUTRI) from Health Science Center (CCS)/Federal University of Recôncavo of Bahia (UFRB). Along with the water collection, a structured questionnaire based on the Sanitation Manual of the National Health Foundation (FUNASA) with 16 questions regarding the water source and its storage (Brazil, 2014).

Bacteriological analysis

For the analysis of total coliforms and *Escherichia coli*, a 100 mL aliquot of each sample was transferred to a sterile first-use bag Twirl'EM (Labplas™ - Montreal, Quebec, Canada). Then, Readycult Coliforms 100 (Merck KGaA™ - Darmstadt, Germany) was added and homogenized until the lyophile was completely dissolved. Samples were incubated in bacteriological oven at 35±1°C for 24±2 h with subsequent reading. All samples positive for *E. coli* in the ultraviolet light test (366 nm) underwent the indole test with the addition of Kovacs reagent (Laborclin® - Pinhais, Paraná, Brazil). For quantification of heterotrophic bacteria, a dilution of 10^{-1} with 0.9% NaCl was initially performed. 1 mL of the dilution was inoculated into a Petrifilm Aqua Heterotrophic Count Plate (AQHC, 3M Company™ - Maplewood, Minnesota, USA). After complete gel solidification, the plates were incubated in bacteriological oven at 36±1°C for 44±4 h. Results were expressed in log CFU/mL (APHA, 2012).

Parasitological analysis

For investigation of parasitic forms in water sources sampled, the methods of direct examination and modified Faust were applied in triplicate. Samples were considered positive when at least one parasitic form was found in one of the methods. For the direct examination, 250 mL of the sample were maintained under spontaneous sedimentation for 24 h at room temperature. The sediment was collected, stained with Lugol's iodine and visualized under optical microscopy (Olympus-Tokyo, Japan) at 100 and 400× (Neves et al., 2016; Teixeira et al., 2016).

For modified Faust, a new aliquot of 50 mL was removed from the spontaneous sedimentation of the total sample and centrifuged at 838 relative centrifugal field (RCF) for 1 min (SPLABOR-Presidente Prudente, São Paulo, Brazil). The supernatant was discarded and the sediment resuspended with 10 mL of sterile distilled water and further centrifuged for 1 min. The supernatant was discarded and the sediment resuspended with 10 mL of zinc sulfate solution (density 1.18 g/mL; Synth-Diadema, São Paulo, Brazil) with further centrifugation at 838 RCF for 1 min. The membrane formed on the liquid's surface was removed with a bacteriological loop, stained with Lugol's iodine (Dinâmica-Indaituba, São Paulo, Brazil) and visualized under optical microscopy at 100 and 400× (Neves et al., 2016; Teixeira et al., 2016).

Physical and chemical analyses

For the physical and chemical analyses, pH, temperature, dissolved oxygen and salinity analyses were performed on field, immediately after water collection with a multiparameter meter AK88 (AKSO®-São Leopoldo, Rio Grande do Sul, Brazil), using an approximate aliquot of 100 mL. Turbidity analysis was performed in laboratory, with a AP-2000 (PoliControl®-Diadema, São Paulo, Brazil) microprocessed bench turbidimeter, with 860 nm wavelength. Apparent color analysis was performed with visual colorimeter DLNH-100 (DelLab®-Araraquara, São Paulo, Brazil) (APHA, 2012).

Statistical analysis of data

Data were processed and analyzed using Statistical Package for the Social Sciences (SPSS) version 23 (International Business Machines™-New York, New York, USA). A normality test (Kolmogorov-Smirnov) was carried out with all quantitative variables. Descriptive and analytical statistics, such as median, maximum, minimum, percentage distribution, Spearman's correlation coefficient, Pearson's chi-squared test and variance analysis (ANOVA) were performed. The adopted level of significance was 5% ($p<0.05$).

Ethical considerations

This study was evaluated and approved by the Human Research Ethics Committee from the Federal University of Recôncavo of Bahia (UFRB) (CAAE: 04022312.0.0000.0056 - Authorization 1.167.637), in accordance to National Health Council (CNS)

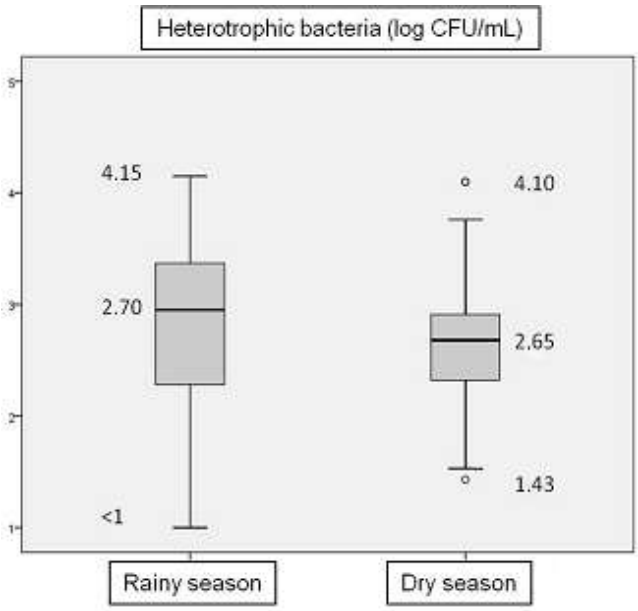

Figure 1. Quantification of heterotrophic bacteria.

Table 1. Total coliform and *Escherichia coli* analysis.

Parameter	Rainy season (N = 53)				Dry season (N=34)			
	Positive		Negative		Positive		Negative	
	n	%	n	%	n	%	n	%
Total coliforms	45	84.9	8	15.1	30	88.2	4	11.8
Escherichia coli	36	67.9	17	32.1	24	70.6	10	29.4

N: Total population; n: sample size; %: percentage.

Resolution 466/2012 (Brazil, 2012). All participants were informed of the study objectives and those who agreed were invited to sign the informed consent form.

RESULTS

Water from every household came from an alternative source of supply, that is, with underground or surface collection, with or without pipeline and without distribution network (Brazil, 2011). Counts of heterotrophic bacteria resulted in maximum values of 4.15 and 4.10 log CFU/mL and minimum values of <1 and 1.43 log CFU/mL by the end of the rainy and dry season, respectively. There were no significant differences between the results of heterotrophic bacteria for both samples (p = 0.071) (Figure 1). Dry season presented a higher proportion of positive samples, with 88.2% (n = 30) positive samples for total coliforms and 70.6% (n = 24) for *E. coli* (Table 1).

No parasitic structures were found in the majority of samples (60.4 and 76.5%). Unidentified protozoa cysts, acari, flagellate protozoa, *Giardia* species and *Endolimax nana* were found in the positive samples, in descending order (Table 2).

Amongst the physical and chemical parameters, there were no significant differences between the means of the two seasons of pH (p = 0.338) and dissolved oxygen (p = 0.859). However, there were significant differences between temperature (p = 0.034), turbidity (p = 0.002) and salinity (p = 0.015) means. It was not possible to perform statistical analysis in the parameter color, because the second water collection was constant, presenting zeroed values. However, there was a significant reduction of this parameter between the rainy and dry season (Figure 2).

Ordinance MS 2914/2011 (Brazil, 2011) subdivides maximum values of potability between allowed and recommended. If a sample is out of the recommended samples, it does not mean it is unsafe for human consumption. However, the source of the problem must be investigated and fixed.

Heterotrophic bacteria, total coliforms and pH have maximum recommended values, however, the presence of *E. coli, Giardia* spp. or *Cryptosporidium* species turbidity and apparent color have maximum allowed

Table 2. Parasitological evaluation.

Parasitological analysis	Rainy season (N = 53)		Dry season (N = 34)	
	Number of findings*			
	n	%	n	%
Negative	32	60.4	26	76.5
Giardia spp.	3	5.7	0	0
Endolimax nana	2	3.8	0	0
Unidentified protozoacyst	15	28.3	3	8.8
Flagellate protozoa	4	7.6	0	0
Acari	0	0	5	14.7

N: Population size; n: sample size; %: percentage; *In a positive sample, one or more protozoa may have been found.

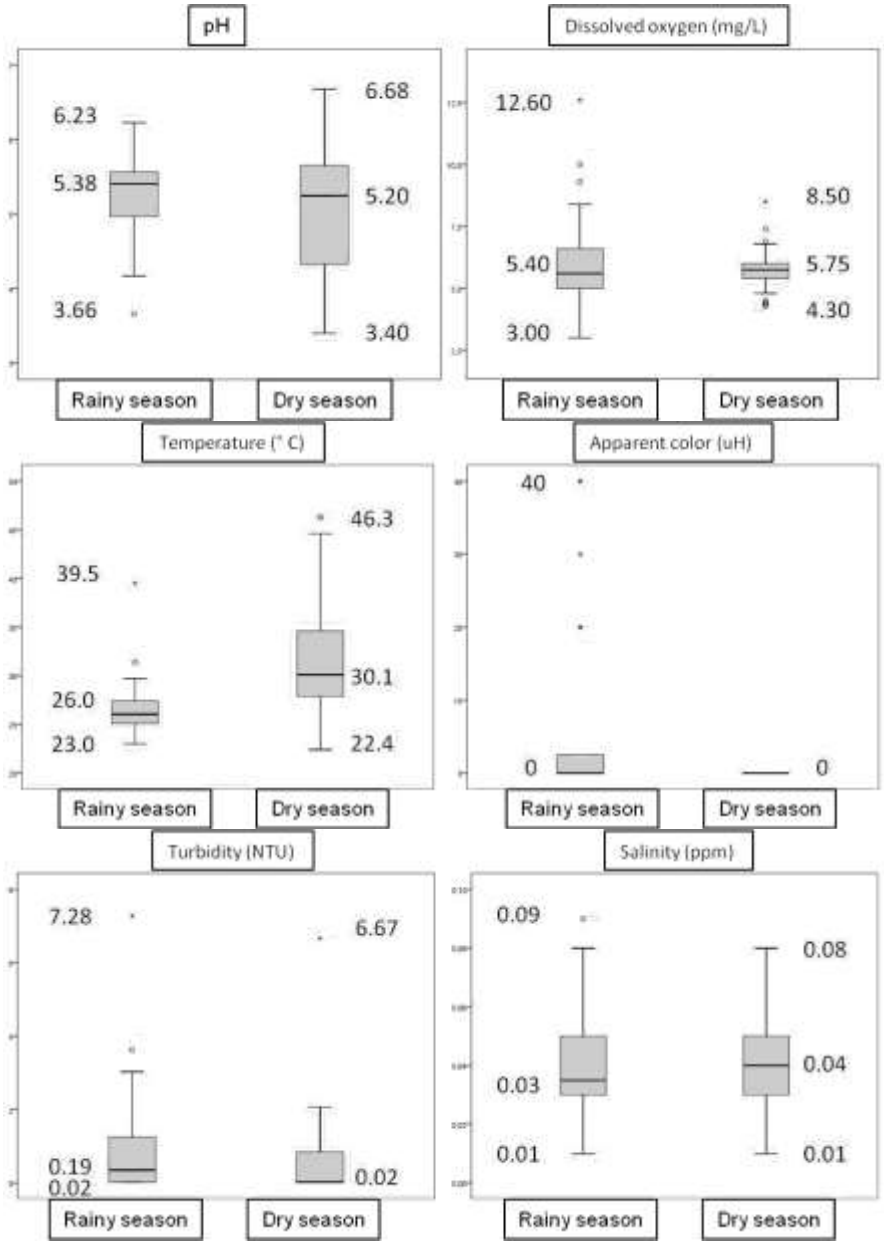

Figure 2. Physical and chemical analysis.

Table 3. Conformity distribution of samples of water intended for human consumption, according to Ordinance MS 2914/2011.

Parameter		In accordance		Not in accordance	
Rainy season (N=53)	**Reference standard**	**n**	**%**	**n**	**%**
Heterotrophic bacteria (log CFU/mL)	≤2.7[x]	27	51	26	49
Total coliforms	Absence in 100 mL[x]	8	15.1	45	84.9
Escherichia coli	Absence in 100 mL[y]	17	32.1	36	67.9
Presence of *Giardia* spp. or *Cryptosporidium* spp.	Absence in 100 mL[y]	50	94.3	3	5.7
pH	Between 6.0 and 9.5[x]	4	7.5	49	92.5
Apparent color (uH)	≤15[y]	47	88.7	6	11.3
Turbidity (NTU)	≤5[y]	51	96.2	2	3.8
All parameters allowed	-	17	32.1	36	67.9
All parameters allowed and recommended	-	0	0	53	100
Dry season (N=34)					
Heterotrophic bacteria (log CFU/mL)	≤2.7[x]	19	55.9	15	44.1
Total coliforms	Absence in 100 mL[x]	4	11.8	30	88.2
Escherichia coli	Absence in 100 mL[y]	10	29.4	24	70.6
Presence of *Giardia* spp. or *Cryptosporidium* spp.	Absence in 100 mL[y]	34	100	0	0
pH	Between 6.0 and 9.5[x]	4	11.8	30	88.2
Apparent color (uH)	≤15[y]	34	100	0	0
Turbidity (NTU)	≤5[y]	33	97.1	1	2.9
All parameters allowed	-	10	29.4	24	70.6
All parameters allowed and recommended	-	1	2.9	33	97.1

x: Recommended values; y: values allowed; N: population size; n: sample size; %: percentage.

Table 4. Spearman's correlation coefficient between bacteriological, physical and chemical analysis.

Parameter	Heterotrophic bacteria	
	Rainy season	**Dry season**
pH	0.134	-0.372[a]
Dissolved oxygen	0.283[a]	0.018
Temperature	0.210	0.102
Apparent color	0.548[b]	*
Turbidity	0.554[b]	0.255
Salinity	-0.108	-0.307

[a]The correlation is significant at the 0.05 level; [b]The correlation is significant at the 0.01 level; *constant.

values. 32.1% samples (n = 17) collected during rainy season were in accordance with the permitted parameters. However, none of the samples was in accordance with the permitted and recommended standards. 29.4% samples from dry season (n = 10) were in accordance with the standards, but only one sample (2.9%) was classified as allowed and recommended (Table 3).

Spearman's correlation coefficient analysis between bacteriological, physical and chemical analyses showed a weak, directly proportional and significant correlation between dissolved oxygen in the results from rainy season (p = 0.040); a moderate, directly proportional and significant correlation between apparent color and turbidity in the results from rainy season (p = 0.000); and a weak, inversely proportional and significant correlation between pH in the results from dry season (p = 0.030) (Table 4).

Results from the parasitological examination presented statistically significant differences between results of heterotrophic bacteria analysis (p = 0.012), with a higher proportion of negative parasitological results in samples in disagreement with the established parameters for heterotrophic bacteria. The other variables are independent (Table 5).

E. coli and heterotrophic bacteria presented statistically significant differences between the distributions of various sources of water (p = 0.01; p = 0.002) and construction of the tank (p = 0.02; p = 0.01). On the other hand, only results from heterotrophic bacteria presented

Table 5. Percent distribution of the bacterial analysis according to the results from the parasitological examination.

Microbiologic		Parasitologic		
		Positive	Negative	p value[1]
Heterotrophic bacteria	In accordance(%)	18.4	28.7	0.012[a]
	Not in accordance(%)	8.0	44.9	

1: *p* value result from the Pearson's chi-squared test; a: statistically significant differences (p<0.05); %: percentage.

Table 6. Percent distribution of *Escherichia coli* and heterotrophic bacteria analysis, according to the results from the verification list.

Parameter	*Escherichia coli*			Heterotrophic bacteria		
	In accordance (%)	Not in accordance (%)	p value[1]	In accordance (%)	Not in accordance (%)	p value[1]
Water source						
Shallow well	12.5	51.7	0.01[a]	25.3	38.0	0.002[a]
Semi artesian well	29.6	17.2		27.5	9.2	
Construction of the tank						
Dweller	13.8	54.0	0,02[a]	27.6	40.2	0,01[a]
Government	17.2	15.0		25.3	6.9	
Openings or cracks at the water tank						
Present	11.5	31.0	0.487	11.5	31.0	0.001[a]
Absent	19.5	38.0		41.4	16.1	
Sewage destination regarding the water source						
Above	11.5	48.3	0.004[a]	24.1	35.6	0.004[a]
Same level	19.5	0.7		28.7	11.6	
Reservoir cleaning time						
Less than six months	17.2	28.7	0.229	32.1	13.8	0.003[a]
More than six months	13.8	40.3		20.7	33.4	
Water reservoir in the household						
Present	18.4	34.5	0.423	35.7	17.2	.,004[a]
Absent	12.6	34.5		17.2	29.9	

1: *p* value result from the Pearson's chi-squared test; a: statistically significant differences (p<0.05); %: percentage.

statistically significant differences between the distribution of variables openings or cracks in the water source (p = 0.001), sewage destination regarding the water source (p = 0.004), reservoir cleaning time (p = 0.003) and presence of a reservoir in the household (p = 0.004) (Table 6).

DISCUSSION

Every household studied had alternative water supply solutions, either individually or collectively. Therefore, it is necessary for the Municipal Health Department to control and monitor the water quality (Brazil, 2011).

A high percentage of improper samples (67.9%) and not recommended (99%) for human consumption was found. Some samples presented 28 times (4.15 log CFU/mL) the maximum recommended for heterotrophic bacteria based on Brazilian legislation (2.70 log CFU/mL). It has been observed that 51% samples were above this reference standard during the first water collection and 44.1% in the second.

Heterotrophic bacteria constitute an indirect indicator of water safety, not identifying the microorganisms, which may be pathogenic or from the water microbiota (Brazil, 2006; Richards et al., 2018). Its high count may indicate bacterial colonization, water treatment ineffectiveness and even a formation of biofilms in the distribution system, from the presence of organic matter in water (Bargellinia et al., 2011; Chowdhury, 2012; Richards et al., 2018).

Sudden changes or values above the potability standards should be investigated in order to identify irregularities and measures must be taken to restore the integrity of the distribution system, such as the maintenance of a minimum free residual chlorine content of 0.5 mg/L (Brazil, 2011).

Ordinance MS 2914/2011 recommends the absence of total coliforms and determines the absence of *E. coli* in 100 mL of water (Brazil, 2011). The total coliforms group comprises bacteria of both enteric and non-enteric origin (Madigan et al., 2016). Therefore, this parameter is not considered a good sanitary indicator of water intended for human consumption, not of crude water, meaning the presence of the bacteria in crude water does not indicate that water is unsafe for consumption (Cabral, 2010).

In order to differentiate enteric coliforms from total coliforms, the thermotolerance characteristic of enteric coliforms is used. *E. coli* is considered the main thermotolerant coliform, being an ideal sanitary indicator in the bacteriological analysis of the water, since its presence indicates that fecal contamination occurred and, consequently, there is a potential risk of presence of other microorganisms of enteric origin, including pathogenic (Cabral, 2010; Brunkard et al., 2011; Madigan et al., 2016). Several strains of *E. coli* are known to be pathogenic to humans. They may present virulence genes, which are involved in colonization, adhesion, and host invasion; pathogenic tissue processes; and avoidance mechanisms (Mainil, 2013).

Parasitic structures were identified in 33.3% of the total samples. Of these, three samples were contaminated by pathogenic protozoa (*Giardia* spp.) and two by commensal protozoa (*Endolimax nana*).

Giardia duodenalis, the protozoa responsible for causing giardiasis, may lead to diarrhea, but most cases are oligosymptomatic or asymptomatic. In Brazil, even with high prevalence of giardiasis (12.4 to 50%), researches of these protozoa are scarce in alternative sources of supply. Its main form of transmission occurs by ingestion of water contaminated by cysts. Population clusters without basic sanitation and sanitary preparation tend to increase the spread of this pathogen. Even with treatment, it is still possible to find cysts in water, due to their resistance to chlorination, filtration and temperature increase (Fregonesi et al., 2012; Santana et al., 2014).

As with *Giardia* spp., protozoa *E. nana* presents fecal-oral contamination through food or water, but is not pathogenic to humans and can be eliminated by conventional water treatment (Poulsen and Stensvold, 2016). The presence of commensal parasites in water, due to their life cycle, can be used as an indicator of fecal contamination, just like *E. coli* (Xavier et al., 2011; Poma et al., 2012).

Free-living protozoa and heterotrophic bacteria are subjected to disharmonious interspecific relations of predation and interspecific competition (Ricklefs, 2016). This fact may be related to the greater number of samples with higher quantifications of heterotrophic bacteria when the result of the parasitological examination was negative.

Due to the lack or inefficiency of sewage collection systems, water can become a vehicle and source of diseases when transporting pathogens. Generally, high levels of parasitic diseases and cases of foodborne diseases prevail where the socioeconomic conditions of the population are more precarious. Less-favored classes are more affected by food contamination due to difficulty or lack of access to basic sanitation, treated water, education and safe food (Andrade et al., 2010; Neves et al., 2016).

Regarding the physical and chemical parameters, apparent color had the highest standard deviation due to the amplitude of its results. As most of the water samples came from shallow wells and it did not underwent filtration, turbidity interfered with the values, increasing them due to suspended particles, thus not demonstrating the actual color of the samples. However, Ordinance MS 2914/2011 establishes apparent color instead of true color as an organoleptic standard of potability, with a maximum value of 15 uH (Scorsafava et al., 2010; Daneluz and Tessaro, 2015).

Turbidity was the parameter with the highest number of samples in conformity (96.2 and 97.1%). These values reflect the low presence of suspended solids, such as inorganic particles and organic debris. High values may be related to the presence of metals in water, such as iron, from the soil itself or from precarious conditions of pumps and plumbing (Adolfo Lutz Institute, 2008; Satake et al., 2012; Daneluz and Tessaro, 2015).

pH was the parameter with the lowest number of samples (7.5 and 11.8%) within the range of recommen-

ded values (6.0 to 9.5), presenting a more acidic pH. Temperature, salinity and dissolved oxygen are not described as potability standards in Ordinance MS 2914/2011; however, they have a direct relationship with bacterial multiplication. The decrease of dissolved oxygen levels is related to decomposition of organic matter (Araujo et al., 2011; Brazil, 2011). Dissolved oxygen reached maximum levels of 15.40 mg/L and minimum levels of 3 mg/L. Temperatures recorded were within the standards for mesophilic microorganisms (minimum of 23.0°C and maximum of 39.5°C). Salinity remained in a range of low values, with minimum of 0.01 ppm and maximum of 0.08 ppm.

Temperature presented significantly higher mean values in the dry season. However, turbidity and salinity presented significantly lower mean values in the dry season. This can be explained by the existence of openings or cracks in most shallow wells, allowing rainwater to conduct soil and organic matter to the wells located in abysses, increasing the amount of organic and inorganic particles in suspension. As a result of the increase in organic matter in the medium, there is an increase of heterotrophic bacteria, since they are chemotrophic, heterotrophic and organotrophic organisms.

Similar bacteriological, parasitological, physical and chemical results were found in other studies with water from shallow and semi-artesian wells in rural regions of Paraná, Rio de Janeiro, Minas Gerais and several cities in the state of São Paulo. These studies have small variations in the quantifications of potability standards, however, the results are unanimous, showing that most samples were unfit for human consumption (Dias et al., 2008; Menezes et al., 2009; Scorsafava et al., 2010; Araujo et al., 2011; Satake et al., 2012; Daneluz and Tessaro, 2015).

Heterotrophic bacteria will consume the oxygen dissolved in water, due to the aerobic respiration process. From this consumption, oxygen levels fall and consequently there will be a greater presence of facultative aerobic and anaerobic bacteria (Santos et al., 2008). Microorganisms are sensitive to pH changes, since the optimum pH ranges from 6.5 to 7.5 for most bacteria, thus lower values can inhibit or delay bacterial multiplication (Machado et al., 2012; Daneluz and Tessaro, 2015).

From the comparison of the bacteriological analyses with the questionnaire, it was possible to identify that results of *E. coli* and heterotrophic bacteria had significant differences between several distributions of the variables.

Water from shallow wells, tanks constructed by dwellers and sewage destination above the level of the water source had higher percentage distributions of samples in disagreement regarding *E. coli* and heterotrophic bacteria.

Results of heterotrophic bacteria also showed significant differences between the variables, in which the presence of openings or cracks in the water source, cleaning time of the reservoir superior to six months and absence of reservoir in the household had larger distributions of samples in non-conformity. Relationships between the other variables were not identified, thus they were considered independent.

FUNASA recommends a minimum distance of 15 m between the wells and the septic tank, and sewage destination should not be at the same level or above the water source (Brazil, 2006). It is also recommended to position breeding farms far from the sources (> 45 m), and both the reservoir and the water source should always remain covered. Finally, reservoirs must be cleaned every six months (Brazil, 2006; Capp et al., 2012; Scalize et al., 2014).

Overall, the study carried out from bacteriological, parasitological, physical and chemical analyses, in association with the verification list, points out to the nonconformity of 99.9% samples from a bacteriological, physical and chemical point of view, considering the MS Ordinance 2914/2011. Therefore, the consumption of this water represents a risk to the population's health of that area, and may lead to the involvement of food borne diseases.

Considering that this rural community is deprived of treated water supply by the state water and sanitation company, alternative and effective measures of water treatment must be taken, such as filtration for the correction of physical, chemical, bacteriological and parasitological parameters, as well as the use of chlorination, or even a solar water disinfection system (SoDiS) in order to eliminate pathogenic microorganisms.

Corrective measures, such as the presence of water reservoirs in the households, protection against animals in the water source, elimination of septic tanks and animal breeding sites near the source, can minimize this risk and guarantee the health of the community.

It is necessary to increase the presence of state with surveillance and quality control of water for human consumption. Sanitary education actions, regular evaluation of potability standards of wells and households, correction of detected failures related to the quality of supply and maintenance of the articulation between the departments of health of the municipalities, states and the union are measures that can be taken to reverse the current situation.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

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Identification of endophytic fungi from roots of two *Dendrobium* species and evaluation of their antibacterial property

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Among all orchids, *Dendrobium* sp. is considered to have high medicinal value. *Dendrobium moniliforme* and *Dendrobium transparens* have immense pharmaceutical, commercial potentiality. However, their fungal endophytes remain unexplored. Isolation and identification of thirteen species of endophytic fungi from the root of *D. moniliforme* as well as five species from the roots of *D. transparens* were done. The two endophytic fungi namely *Aspergillus flavus* and *Trichoderma harzianum* were common for both plant species. Antimicrobial assay was done against selected human pathogen both gram positive and negative *Escherichia coli, Pseudomanas aeruginosa, Staphyllococcus aureus* and *Staphyllococcus epidermidis*. The antimicrobial assay showed the significant role of endophytic fungi to inhibit the bacterial growth. Methanolic extract of DeMon VI and DeMon X were prepared to identify the bioactive compounds. Phenolic and 3-Eicosene,(E)-compounds were identified from extract of DeMon VI whereas Pentadecanoic acid, 14 methyle-, methyl ester and Diethyl Phthalate were identified from extract of DeMon X.

Key words: Orchids, *Dendrobium* sp., endophytic fungi, Human pathogen.

INTRODUCTION

Orchids are the most fascinating group of flowering with immense advantage and shows diversity in distribution (Pant and Raskoti, 2013; Pant et al., 2017). They are mostly found in moist and shady place, some are lithophytes, saprophytic and even terrestrial. These orchids have both ornamental as well as a medicinal value, used in traditional medicine to cure different diseases. Most of the orchids are listed on the Convention on International Trade in Endangered Species (CITIES) Appendix II, its exploitation is still carried on (Pant and Raskoti, 2013). In Nepal, the orchid are under threats and at verge of extinction (Subedi et al., 2013). The endophytic fungi resides in the plant tissue without causing harm to host plant and protect against pathogenic attacks by means of secondary metabolites (Schulz, 2006). Fungal endophyte provides variety of secondary bioactive products that have wide application in medicine, agriculture and industry (Selim et al., 2012).

Dendrobium is the second largest genus of orchid, about 30 species are found in Nepal. Most of these species have medicinal property but are verge of extinction. Recent report explores the antibacterial activity

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of the Dendrobium plant extract. In the present study, two *Dendrobium species*, *D. moniliforme* and *D. transparens* have been selected. These species are naturally found in central hills of Nepal. *D. moniliforme* are typical epiphytic orchid with white petal and semispherical anther (Xiaohua et al., 2009; Pant et al., 2016). The extract of *D. moniliforme* has significant role in curing osteoporosis and bone fracture (Baek et al., 2016). It has been used in traditional medicine. D. transparens is a typical epiphyte with graceful medium size flower. Flower has white petal with purple blotch at the tip (Sunitibala and Kishor, 2009). The medicinal properties of the *D. transparens* have not been studied yet. However, the reports suggest that it is used to cure various diseases by local people in least developed countries.

The multi drug resistance bacteria have become a threat to the public health and challenge to the scientific community. Over the years, human pathogenic bacteria have developed the mechanism to deal with various antibiotics. The multidrug resistant bacteria have become a global threat as these bacteria shows resistant to broad spectrum antibiotics. As a result, mutltiple drug resistant (MDR) bacteria spreads different diseases causing high mortality, high healthcare cost (Köck et al., 2010; Mongalo et al., 2013). Report suggest, the Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi* and *Acinetobacter baumannii* have developed the mechanism to resistant to most all antibiotics (Chambers, 1997; Kim et al., 2005; Patzer and Dzierzanowska, 2007).

This study highlights the isolation and identification of endophytic fungi from the roots of the selected *Dendrobium* species. The study focus on the antimicrobial activities of isolated and identified fungi against selected human pathogen. These studies explore the alternate method to tackle the multidrug resistant bacteria. In this regard, the human pathogen that were selected for antimicrobial assay are *E. coli, P. aeruginosa, S. aureus* and *Staphylococcus epidermidis.*

MATERIALS AND METHODS

The selected two *Dendrobium* species were collected from the two different places of the Central Nepal. *D. moniliforme* was collected from dense forest of Daman, Makwanpur district at an altitude of 1400-1600 m and the host tree was *Quercus semicarpifolia*. *D. transparens* was collected from the dense forest of Suryabinayak, Bhaktapur at an altitude of 1500-1600 m and the host tree was *Rhododendron* species.

Isolation of endophytic fungi from wild roots

A sterile scalpel was used to take the healthy roots from the three different epiphytic orchids. For the fungal isolation, method followed was described by Porras-Alfaro and Bayman (2007). First the roots were washed with running tap water for 20 min, 75% ethanol for 1 min, 3% Sodium hypochlorite (NaOCl) for 3 min and rinsed with sterile water for 3 times. Then the roots were cut into 1 cm pieces. 5 pieces of roots were placed in 15 petri plates containing Potato dextrose agar (PDA) supplemented with antibiotics as chloramphenicol 50µg ml^{-1} kept in room temperature (25-27°C) at dark for 3-7 days. The fungi growth were observed with their different colony pattern and were sub-cultured to obtain pure culture based on colony pattern. For the conservation of fungi, it was stored in freeze at 4°C until use.

Identification of fungi from root of selected wild *Dendrobium* species

Each pure colony obtained was used for the identification of fungal strain to genus and or species level on cultural characteristics and some morphological traits as overse and reverse colony colour, colony texture and the growth patterns. The microscopic characterization of strain was based on observation through microscope. Specimens for light microscope was mounted in lactophenol - cotton blue for observation of spores and sporulating structure and photograph were taken. Fungi were identified with the help of monographs, literature and following books: Watanabe 2010 and website Mycobank.org

Collection of bacterial strain

The bacterial strain used for the assay of antibacterial activities of orchid fungal endophytes was human pathogenic bacteria. *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) were gram negative bacteria whereas *S. aureus* (ATCC 259263) and *S. epidermidis* (ATCC 12228) were gram positive bacteria. These bacterial strains were identified and obtained from Shukraraaj Tropical and Infectious Disease Hospital, Kathmandu, Nepal.

Biochemical analysis of two fungal extract

Fungi were grown in 300 ml of Cazpek broth for 15 days in the rotator shaker. The supernatant was obtained after centrifugation of the fungal broth at 5000 rpm for 30 min and then further filtered with the help of Whatman filter paper 1. The pH of the filtrate was made acidic by adding few drops of 5% sulphuric acid. Further, the three consecutive wash with equal volume of ethyl acetate was done. The organic portion, ethyl acetate extract was then collected with help of separating funnel. The residue of crude extract was obtained by subjecting the ethyl acetate extract to the rotary evaporator at 40°C. The residue was then suspended in 2 ml of High-performance liquid chromatography (HPLC) grade methanol. The bioactive compound was identified with the help of GCMS-QP2010 Ultra instrument fitted with RTX-5MS (30 m x 0.25 x 0.10) column. The various parameters was considered such as initial temperature of 100°C for 1 min and the final temperature of the oven 25°C. Rate of Helium flow of the instrument was 1 ml/min and the ionization voltage was 0.80 KV. The sample injection was in split less mode. Mass spectral scan range was set at 30 to 600 (m/z). The peak obtained for each compound in the graph was analyzed by comparing with the library of National Institute of Standard and Technology, NIST, US.

Antibacterial assay

The antibacterial test was performed by radial streaking method. In this regard, dual culture technique was followed. Each PDA plate consisting 1cm of fungi disc at center was incubated for three days at temperature of 25°C in dark condition and same plate was used for the antibacterial test. The different strain of bacteria

was taken which were streaked with the help of cotton swap from the periphery of plate towards center up to the end of colony of fungi. After swapping, the plates were kept in the incubator at 30°C for 24 h and results were observed. The experiment were repeated independently for three times. For the control, gentamycin disc of 10 mg was used, which was kept at the center of the petri plate and all the selected bacterial strain were streak from the radial ends of plate and incubated at 30°C for 24 h and the results were noted.

Measurement of zone of inhibition (ZOI)

After 24 h of incubation at 37°C, the plates were observed. The antibacterial test was evaluated as the presence or absence of inhibition zone. The clear distance between fungus and bacteria was zone of inhibition and measurement was taken in mm by the help of the scale. The width of inhibition zones between the pathogen and the endophytes was evaluated as >10 mm (strong inhibition), 2-10 mm (moderate inhibition) and <2 mm (weak inhibition) (Paul et al., 2007). For positive control, the inhibition zone was measured following same procedure as with fungi.

Statistical analysis

The results presented are the means of the three independent replicates ± standard error of mean (S.E.M). The one way ANNOVAs was done to find significant level of the data at the level of $P< 0.01$ and analyzed with the help of Microsoft Excel.

RESULTS

Identification of isolated fungi

Identification was carried out on the basis of macro-morphology and micro-morphology. Macro-morphology includes overse and reverse colony colour, colony texture and growth rate of colony whereas micro-morphological study includes conidia size, shape, mycelium, spore size and shape through photograph taken. Fungi were identified with the help of available literature, photograph and monograph method and spore structure was determined. A total of sixteen endophytic fungi species were isolated and identified, out of which thirteen strains were isolated from *D. moniliforme* and five strains from *D. transparens.* Interestingly, two species were common in both species. Most dominant species found was *Aspergillus* species and *Fusarium* species. *Aspergillus flavus*, *Aspergillus clavatum*, *Aspergillus brevipes*, *Aspergillus fumigatus*, *Hypoxylon fragiforme*, *Aspergillus niger*, *Colleotrichium* sp., *Leptosphaerulina chartarum*, *Fusarium* sp, *Cladosporium* sp., *Fusarium equiseti*, *Trichoderma harzianum* were identified from *Helminthosporium* sp., *Fusarium* sp., and one strain of isolated fungi from *D. transparens* remained unidentified (Figure 1 and Table 1).

Chemical profiling

The chemical profiling of the organic extract of two fungi was done. The organic extract of DeMon X and DeMonVI showed the presence of diverse secondary metabolites. The major compound identified from the Gas Chromatography Mass Spectrometry (GC/MS) analytical technique from the extract of DeMonVI were Phenol, 2,4-bis(1,1-dimethylethyl)-, Eicosene,, Pentadecanoic acid, 3-Octadecene. The major compound identified from theGCMS analytical technique from the extract of DeMon X were Diethyl Phthalate, Pentadecanoic acid, 14-methyl-, methyl ester. The detail of the chemical diversity of the both fungal extract is shown in the GC-MS chromatograph in supporting Figure 1a-p and Table 2.

Antimicrobial assay

The isolated and identified fungi from *D. moniliforme* and *D. transparens* showed antibacterial properties against at least one of four human pathogenic bacteria. All the endophytic fungi isolated from both the species of *Dendrobium* showed strong antibacterial activity against gram positive *S. epidermidis.* However, some endophytic fungi like *A. brevipes, A. fumigatus*, *Colleotrichium alatae*, *L. chartarum*, *Helminthosporium* species and *Fusarium* II species did have significant impact at level of $p <0.01$ on growth inhibition against human pathogenic gram positive *S. aureus.*

Similarly, the endophytic fungi are tested against gram negative bacteria following same method. Almost all fungi isolated have highest zone of inhibition against *E. coli* except *Hypoxylon fragiforme*. Most of the fungi showed the growth inhibition of bacterium *P. aeruginosa* except endophytes *H. fragiforme*, *F. equiseti*. The positive control (gentamycin) showed moderate zone of inhibition against all four different human pathogenic bacteria.

There is significant growth inhibition at the significane level of $p<0.01$ for the selected human pathogen by the orchid fungal endophytes summarized in Figure 2. The figure shows the comparative study of their pathogen growth inhibition activity among the endophytes and with respect to the control. The zone of inhibition of fungi was greater than control gentamycin (10 mg). The results of Table 2 depict the level of the inhibition against the four different bacteria. Nine species of fungi namely *A. flavus*, *A. clavatum*, *A. niger*, *Fusarium I* species, *Fusarium oxysporium*, *Cladosporium tenuissimum*, *Trichoderma*, *Fusarium* II species and one unidentified species have highest antibacterial properties with all four human pathogenic bacteria.

DISCUSSION

Most of the endophytic fungi isolated were *Aspergillus* sp., *Fusarium* sp., *Trichoderma* sp., *Hypoxylon* sp., *Colletotrichium* sp., *Leptosphaerulina* sp., *Cladosporium* sp., and *Helminthosporium* sp. belonging to ascomycetes. *Aspergillus* sp. has been well known for its medicin Pant

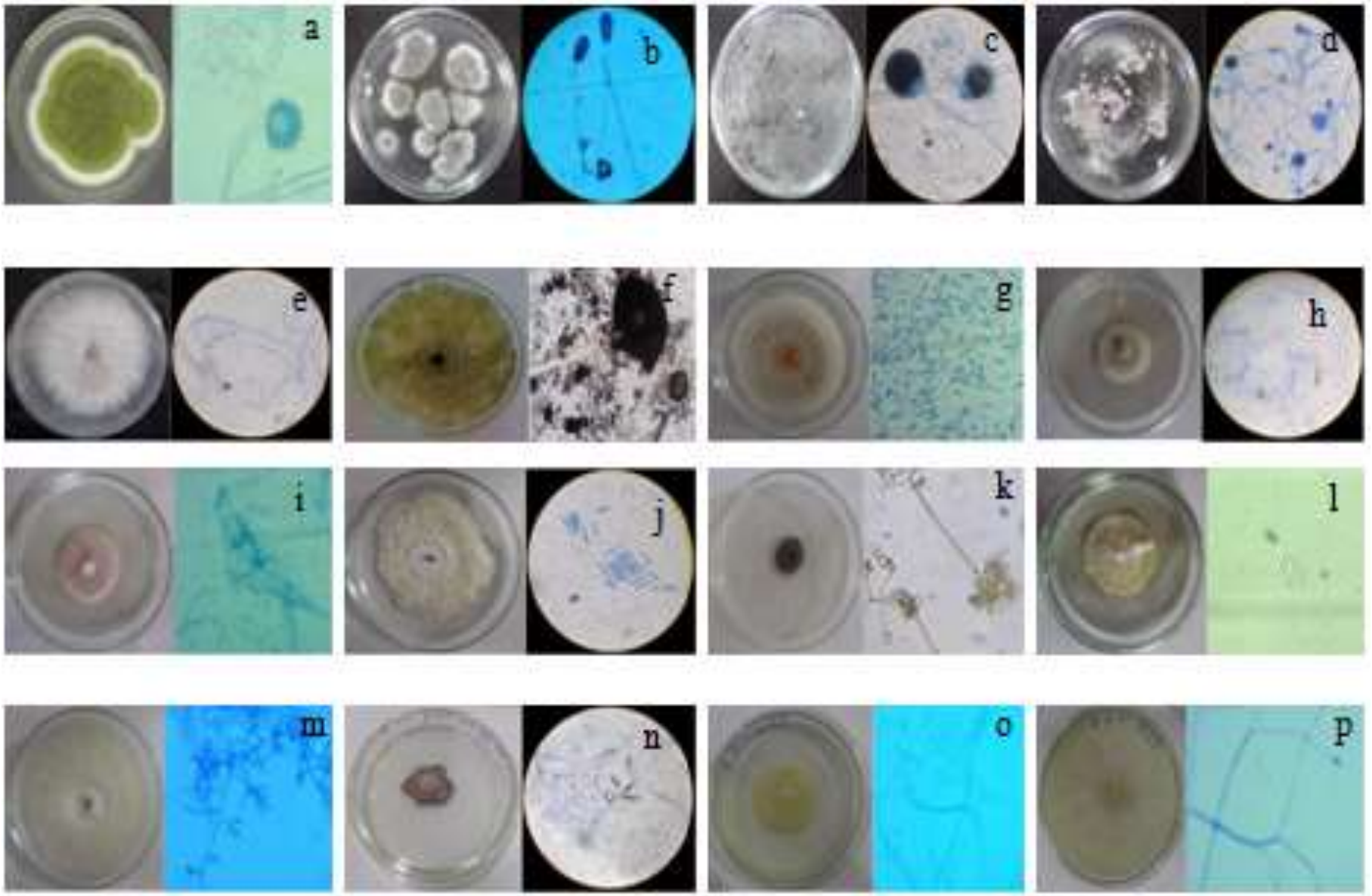

Figure 1 (a-p). Overse colony on PDA and microscopic view at 40X of endophytic fungi. (a)*Aspergillus flavus*, (b) *A. clavatum*, (c) *A. brevipes*, (d) *A. fumigatus*, (e) *Hypoxylon* sp., (f) A. niger, (g) *Colleotrichium* sp., (h) *Leptosphaerulin*a sp., (i) *Fusarium* sp., (j) *Fusarium* sp., (k) *Cladosporium* sp., (l) *Fusarium equiseti*, (m) *Trichoderma* sp., (n) *Helminthosporium* sp., (o) *Fusarium* sp., and (p) unidentified respectively.

Table 1. List of endophytic fungi from roots of selected wild *Dendrobium* species.

Code No. of isolates	Peculiar Characteristics of isolates	Tentative affiliation	Fungal taxonomy
DeMon I/DeTra V	Colony radial growth with grey to yellowish colour. Conidial head radiate and biseriate with globose shaped	*Aspergillus flavus*	Eurotiomycetes
DeMon III	Colony radial growth with grey to green, Conidial head radiate and uniseriate with ellipsoidal	*Aspergillus clavatum*	Eurotiomycetes
DeMon IV	Colony radial growth with grey to green colour. Conidial head uniserate and columnar with globose shaped	*Aspergillus brevipes*	Eurotiomycetes
DeMon V	Colony radial growth with white to greenish on maturation. Conidial head very short with uniserate and vesicle globose shaped	*Aspergillus fumigatus*	Eurotiomycetes
DeMon VI	Colony radial growth with white to black on maturation. The mycelium was observed which were branched and sepatate no sporulting stage	*Hypoxylon* sp.	Euascomycotina
DeMon VII	Colony radial growth with was brown to black on maturation. Conidial head were biseriate and globose in shape, Vesicles were spherical to globose	*Aspergillus niger*	Eurotiomycetes
DeMon X	Colony radial growth with white to pale orange at the centre on maturation. Conidia were cylindrical with broadly rounded ends	*Colletotrichium* sp.	Sordariomycetes
DeMon XI	Colony radial growth with grayish and become brown. Mycelium was brown and septate.	*Leptosphaerulina* sp.	Dothideomycetes
DeMon XII/XVII	Colony radial growth with white at first and become pink. Conidia are smaller in size and are cylindrical, gradually pointed and curved towards end.	*Fusarium* sp.	Sodariomycetes
DeMon XIII	Colony radial growth with white to pinkish. Conidia was comparatively larger in size gradually pointed and curved towards ends	*Fusarium oxysporium*	Sodariomycetes
DeMon XIV	Colony radial growth with greenish brown on maturation. The hyphae was brown, erect, and septate. Conidiophores was brown may be septate and shows tree like branching	*Cladosporium* sp.	Dothideomycetes
DeMon XVI	Colony radial growth with white to greenish on maturation. The conidia are curvature, tapered and elongated apical cell	*Fusarium equesti*	Sordariomycetes
DeMon XIX/DeTra II	Colony radial growth with white to greenish on maturation. Conidial were globose to sub-globose, flask shaped and arranged in divergent groups	*Trichoderma* sp.	Sordariomycetes
DeTra I	Colony radial growth with grayish brown on maturation. Hyphae was branched, septate, pale brown. Conidiophores was cylimdrical and curvature	*Helminthosporium* sp.	Dothideomycetes
DeTra III/IV	Colony radial growth with white to pale yellow on maturation. microconidia are cylindrical in shape	*Fusarium* sp.	Sordariomycetes
DeTra VI/VII	Colony radial growth with white cottony to pale yellow on maturation. Mycelium baranched aseptate Conidiophore oval or globose shaped	Unidentified	Unknown

oactive compound importance and that have been catalogued as a scientific database (Furtado et al., 2002; Thorati and Mishra, 2017). The most important and well-known compound isocoumarin 7,7′-homodimers and dihydroquinolone derivative have been isolated from *A. versicolor*. The report clearly demonstrates the biological activities such as antimicrobial as well as cytotoxicity. In the present study, *A. fumigates* and *A. niger* that has been isolated and identified were also studied for their antimicrobial activity in the previous study (Furtado et al., 2002; Thorati and Mishra, 2017). A. fumigates is known to produce 3,4-dimethoxyphenol and 1,3,5-trimethoxybenzene (Furtado et al., 2002). In our findings, the *Hypoxlon* sp. was able to inhibit the growth of two pathogen *S. aureus* and *S. epidermidis*. However, the fungi did not show any effect on the growth of *E. coli* and

Table 2. List of the various bioactive compounds identified from fungal extract of DeMon X and DeMon VI.

Peak	Retention time	Area%	Name	Base m/z	Reported biological activities
1	6.261	5.02	Phenol, 2,4-bis(1,1-dimethylethyl)-	191.05	Antibacterial, antifungal and antioxidant properties (Ramanujam et al., 2014)
2	6.823	20.33	3-Octadecene, (E)-	43.05	Antimicrobial property (Guo et al., 2008)
3	7.002	13.10	Diethyl Phthalate	149.25	Antimicrobial property
4	8.307	16.52	3-Eicosene,(E)-	43.10	(Dehpour et al., 2012)
5	9.229	3.09	Petadecanoic acid, 14 methyle-, methyl ester	74.00	Antimicrobial property
6	9.260	45.84	Petadecanoic acid, 14 methyle-, methyl ester	74.15	(Dehpour et al., 2012)

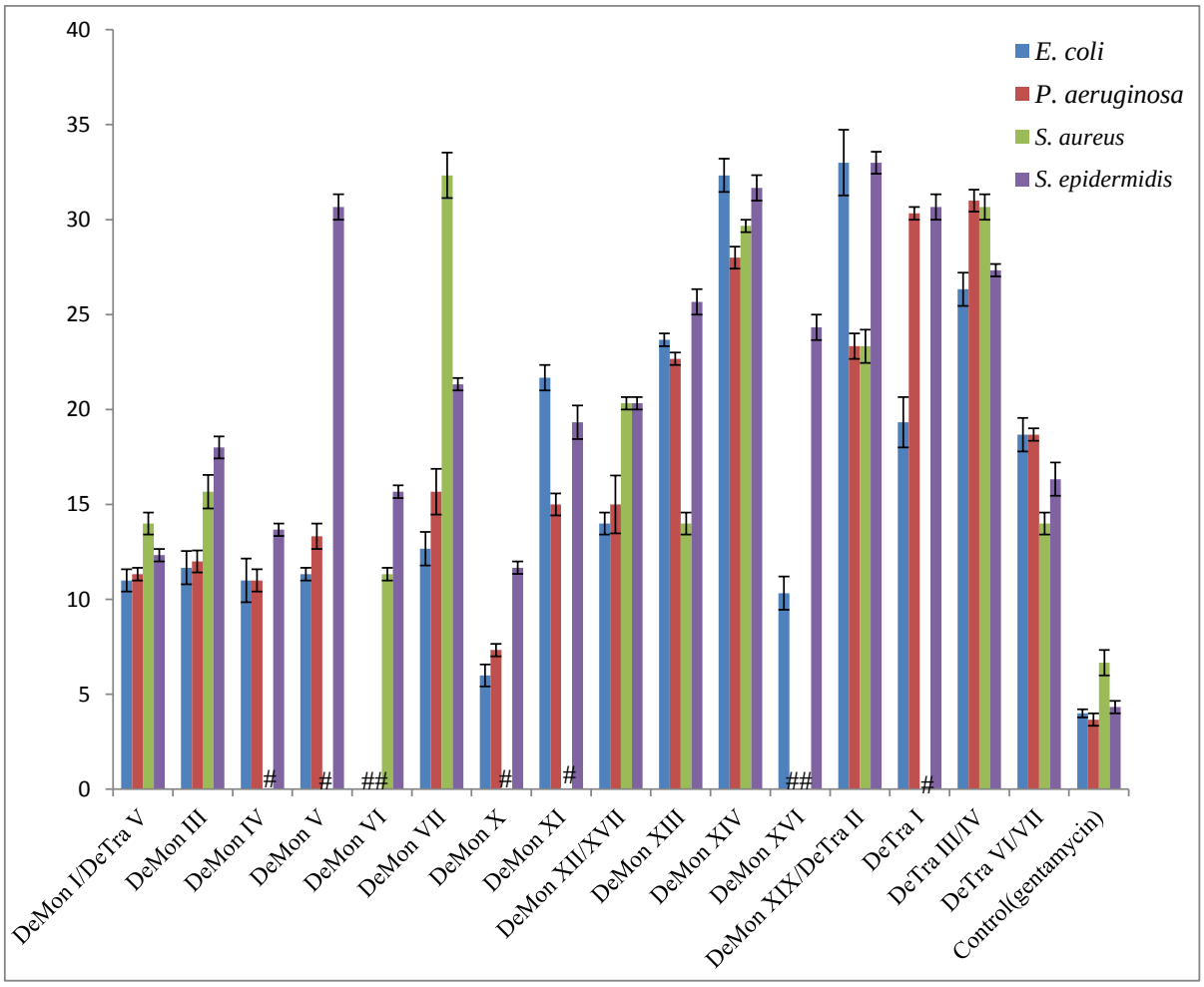

Figure 2. Antibactieral activity of the endophytic fungi against selected humane pathogen. The fungi DeMonIV, DeMon XIX, DeMon XII, DeMonXVII showing high growth inhibition of the pathogen compared to antibacterial activity of other fungi and control.# represent no antibacterial activity of the corresponding fungus. One way ANOVAs was performed for the significant level of $p<0.01$.

P. aeruginosa. The chemical profiling of the *Hypoxlon* sp. DeMon VI showed the presence of Phenol, 2,4-bis(1,1-dimethylethyl)-, Eicosene, Pentadecanoic acid, 3-Octadecene. The compound has been well investigated for their antioxidant and antibacterial activities (Dehpour et al., 2012). Similarly, Colletotrichum sp., also

demonstrate the antimicrobial properties. The organic extract of DeMon X was investigated for bioactive compound. The compounds such as Diethyl Phthalate, Pentadecanoic acid, 14-methyl-, methyl ester were identified from extract. These compounds have both antimicrobial and antioxidant activities. However, the fungus appears to show antibacterial activities but was ineffective against S. aureus. Similarly, DeMon XI isolated from *Dendrobium moniliforme* have been characterized as Leptosphaerulina sp. The DeMonXI was able to show the antibacterial effect against *E. coli*, *P. aureogenosa*, *S. epidermis* but was ineffective against *S. aureus*. However, similar kind of study was done with Endophytic fungi *Leptosphaerulina* sp. isolated from the mangrove plant *Acanthus ilicifolius*. The fungus was reported to produce novel compounds pyranonaphthazarin and 2-naphthoic acid derivatives having strong antimicrobial properties against *S. aureus* (Cui et al., 2017). DeMon XIV was characterized as *Cladosporium* sp. that has shown strong antibacterial activity against all the selected human pathogenic bacteria. In previous study, Phenylacetic acid, p-hydroxyphenylethyl alcohol, and L-beta-phenyllactic DeMonXI was acid were isolated from the extract of Cladosporium sp. that has an antimicrobial property (Ding et al., 2008). The result correlates the presence of such metabolites that have shown higher antibacterial activity in our investigation.

In the present study, *Fusarium* species were relatively isolated in large number. Most of them are known for their antimicrobial, antioxidant activities as well as secondary metabolite production. DeMonXII, DeMonXIII, DeMonXVII and DeMonXVI isolated from *D. moniliforme* and DeTra III and DeTra IV isolated from *D. tranparens*. These fungi were characterized as Fusarium sp. and were able to show antibacterial activity against the selected human pathogen. DeMon XII, DeMonXIII, DeMon XVII, DeTra III and DeTra IV strongly inhibited the growth of all four selected human pathogenic bacteria. However, the isolate DeMonXVI show the activity against *E. coli* and *S. epidermidis*. Simliarly, DeMon XIX and DetraII were characterized as *Trichoderma* species and were able to strongly inhibit the growth of all four selected human pathogenic bacteria. Importantly, a novel L-lysine oxidase enzyme have identified from the fungal extract of *Trichoderma viride* that have antitumor activity. New species of *Trichoderma hypoxylon* produces various secondary metabolites such as trichothecenes and epipolythiodiketopiperazines (Sun et al., 2016). Similarly, the various secondary metabolites have been reported from *Trichoderma* species may have contributed to their antibacterial acitivity. DeTraI which is characterized as *Helminthosporium* species was able to inhibit the growth of all three human pathogens *E. coli, P. aeruginosa,* and *S. epidermidis* expect *S. aureus.* The fungi that remain uncharacterized by macro and micro morphology also showed that significant antibacterial activity against all the human pathogenic bacteria.

Conclusion

This research concludes that large number of fungi belonging to the phylum ascomycetes is reside in the roots of wild *D. moniliforme and D. transparens* species. On the basis of morphological study sixteen species of fungi were identified. Among them thirteen species from *D. moniliforme* and five species from *D. transparens* and two species common in both species. The identified fungi were tested against four different human pathogenic bacteria *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis*. Each fungus shows antibacterial properties against at least one of the four human pathogenic bacteria. Most of the fungi show antibacterial activity against *S. epidermidis*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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