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Influence of Spirulina platensis Cyanobacterium on Some pathogenic bacteria isolated from different diets .

Agriculture

KEYWORDS :Spirulina platensis, pathogenic bacteria,diets

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ABSTRACT

The present work was carried out to examine Spirulina Platenss water extract as antibacterial agent. Twenty bacterial strains (M01 –M20) were isolated under aerobic conditions from twelve traditional food products; further all strains were identified by partial 16SrRNA gene sequencing and phylogenetic analysis. Fifteen strains were identified by 97-100% identity including Bacillus circulance (4), Bacillus subtilis (4), Staphylococcus aureus (3), and Echerichia coli (4). Other isolates were identified by 85-92% identity; therefore these species are considered as new ones and named: Bacillus sp. (M07, M09, M10) and Staphylococcus sp. (M12, M13).Antibacterial activity of S. platensis water extract was recorded against the 20 bacterial strains. At S. platensis water extract concentration of 100mg/ml; inhibition zones diameter for all tested isolates ranged between 4 and 12 mm, while minimum inhibitory concentration of S. platensis extract(1µl/ml)was found to be effective against all Bacillus circulance, Esherichia coli strains and 2 out of 5 strains of Staphylococcus aureus. Results obtained revealed that water extracts of S. platensis showed better antibacterial activity against the pathogens used; further researches are recommended to identify and purify natural bioactive products from S. platensis as antimicrobial compounds, this will provide a new opportunity for the application of this cyanobacterium as a perfect probiotic, neutraceutical and in preventing some chronic diseases where free radicals are involved.

INTRODUCTION

Food-borne diseases are major international health problems and important causes of reduced economic growth (WHO., 2002). Food- and water-borne diarrheal diseases are the leading causes of illness and death in developing countries, killing as estimated 2.2 million people annually; most of them are children (Mensah et al., 2002). Bacteria have accounted for more than 70% of deaths associated with foodborne transmission (Hughes et al., 2007). Food-borne illnesses associated with Staphylococcus aureus , Bacillus cereus, Escherichia coli 0157:H7 and Salmonella enteritidis present a major public health concern throughout the world (Isara et al., 2010). Bacillus species are Gram positive rod, grow under aerobic conditions and spore formers. Bacillus cereus causes two types of food-borne diseases: a diarrheal syndrome caused by complex enterotoxins (diarrheal toxin) and emetic syndrome caused by emetic toxins. Itoccurred in 98% of test minced meat , 60% of sausage , 40% of rice grains , 44% Koshari or ice-cream, 36% of pasteurized milk samples and 90% of raw vegetables as investigated by Saleh (1993) and Kim et al. (2004). E. coli is Gram - negative bacteria, short rod, grow under aerobic conditions and non-spore forming. Outbreaks can infect thousands of people causing bloody diarrhea and hemolytic uremic syndrome (HUS) that can result in severe illness or even death (Chattaway et al., 2011). Higher numbers of outbreaks have been attributed to the consumption of water ,fresh leafy and oods products (Sospedra et al., 2012). Staphylococcus aureus is a Gram-positive cocci, catalase positive , and appear as characteristic 'bunches of grapes'. Consumption of foods contaminated with S. aureuscan cause gastroenteritis, nausea, vomiting, diarrhea, and abdominal pain within 1-6 hrs post-consumption of contaminated foods (FDA., 1998). Meat was a substantial (11.2–25.0%) source of bacterial toxins produced by S. aureus, B. cereus and E. coli (EFSA., 2007). The process of cooking should kill the bacteria but some bad practices of handling or storage can even increase the bacterial load of the initial product.

Spirulina platensisis a microscopic photosynthetic filamentous cyanobacterium that has a long history of use as a safe food, lacking toxicity (Sotiroudis and Sotiroudis, 2013). It is found in a variety of environments; soil, sand, sea water and fresh water, and is consumed as human food supplement for centuries (Mexico 4000 years ago) because of its best known nutritional value. It contains 78% proteins, 4-7% lipids, minerals, vitamins, carbohydrates (Pugh et al., 2001) and some natural pigments (Toyomizu et al., 2001).Today, there are several companies producing Spirulina platensis as a food supplement, which is sold in many health food stores around the world. Worldwide medical research has discovered that S. platensiswith its unique blend of nutrients has helped to compact many health problems like food poising diseases (Bouhlal et al., 2010).

Spirulina platensis has the same properties of probiotic bacteria including; prevention of cancer, protection against many opportunistic human pathogens (Brook, 2005). Another efficiency of Spirulina platensis; its free radical scavenging properties and antioxidant activity, where it contains a number of natural pigment such as chlorophyll, beta-carotene and phycocyanin (Gad et al., 2011). The probiotic effects of these microorganisms include prevention of constipation in elderly people, preventing diarrhea and prevention of cancer (Lee et al., 2007). Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer beneficial health effects (FAO/WHO., 2002). In recent years, there has been an increase in research into probiotics, as well as growing commercial interest in the probiotic food/food supplement concept. Probiotics now comprise a large portion of the functional food market; dairy products, including yogurt, fermented milk and cheese, stay at the front position of probiotic food improvement.

The food industry has a challenge to produce low cost, nutritive and convenient foods. In order to meet this demand, it is important to study sources of fibers and antioxidants compounds that are technology viable, with positive environmental and economic impacts (Bolanho et al., 2014). In recent years, there has been an explosive interest in the use of antioxidants nutritional supplements (Gigante et al., 2007). Epidemiological evidence suggests that intake of some vitamins, minerals, and other food constituents may help to protect the body against heart disease, cancer and the aging process, and that antioxidants may have a

protective effect, either in preventing these diseases or lessening their severity (Hsia et al., 2007).Bacteria have been source for majority of antibiotics and therapeutic compounds. In addition researchers are searching for new antibiotics/therapeutic compounds from a group of microbes that has been overlooked in the past.Cyanobacteria; of which S.platensis has emerged as one of the most promising species for synthesizing potentially new therapeutic compounds; water extracts of spirulina had been reported to possess antibacterial property (Islam et al., 2004).

Recent years molecular methods are progressively used for identification of pathogenic bacteria caused food poising diseases;many of these methods are based on 16S ribosomal DNA sequences (16S rRNA gene) and develop either hybridization or PCR techniques. The methods included 16S r RNA gene sequences can be utilized to place diagnostics into a phylogenetic structure and can be connected to databases providing several thousand sequences that increase day by day (Janda and Abbotte, 2007).

Therefore, the purpose of the present study aimed to isolate and identify some pathogenic bacteria from different diets obtained from some traded restaurants , and studying the efficiency of S. platensis water extracts as anti-bacterial agent.

2. MATERIAL AND METHODS

2.1. Isolation of bacterial strains

Twelve traditional food products were used in the present study as source for isolation of pathogenic bacteria. All samples (50) were collected from local restaurants. According to the standard method of the microbiological examination, streak plate technique was applied in the present study to isolate and purify culture bacterial strains (ICMSF., 1996) in nutrient agar plates. Standard Biochemical tests were performed following standard procedures according to Cappuccino and Sherman (1999).

2.2. Partial sequencing of 16S rRNA gene

2.2.1. DNA extraction from culturable bacteria

The genomic DNA of culturable bacterial isolates was extracted using QIAamp DNA Mini Kit (Qiagene) according to manufacturer'sprotocol.

2.2.2. DNA sequencing

The sequence step was commercially carried out by Macrogen Inc., Seoul, South Korea, through 16S rDNA sequencing (Ahemad and Khan, 2010). The obtained partial nucleotide sequences of the Materials & Methods 35 16SrRNAgene were aligned using Clustal W from MEGA 4.0 software (Tamura et al., 2007) and compared with the homologous sequences of the type strains, available in the GenBank database.

2.3. Preparation of S. platensis water extracts

S. platensis food supplement tablets were obtained from DXN Company which is the first multi-level marketing (MLM) company in Malaysia to produce spirulina from the cultivation process to finished goods, where only the selected best species is naturally cultivated in a clean pond with no pesticides or herbicides are applied.1, 10, 50 and 100 mg of S. platensis tablets were weighed and each concentration was dissolved in 1ml distilled water to obtain four water extracts of S. platensis.

2.4. Antimicrobial activity of S. platensis water extracts

All isolated and identified bacteria were maintained on Muller Hinton broth and agar medium until used for inoculums preparation, bacterial cell suspension which equilibrated their concentration to a 0.5 of McFarland standard turbidity scale (10^5 – 10^6cfu/ml for bacteria) was used.

Antibacterial Assay: 0.5 ml of bacterial suspension was seeded on Muller Hinton agar media. Disc paper of diameter of 2mm was putted on these media plates and filled with 10µl from S. platensis water extracts of concentrations 1, 10,50 and 100 mg/ml distilled water. Distilled water was taken as control; the results were recorded as mean diameter of growth inhibition zone surrounding the disc and compared with control.

Minimum Inhibitory Concentration of S. platensis: Water extracts of S. platensis dry weight of different concentrations were screened against twenty bacterial isolates (M01 to M20). The minimum inhibitory concentration (MIC) was determined only with microorganism that displayed inhibitory zones. Half ml of suspension of each test organism was mixed with agar medium and poured into plate; then S. platensis dry weights of 1, 10, 50,100 mg;each was diluted in one ml of distilled water. For determining MIC,a series of S. platensis dilutions ranged between1 and 50µl /ml per filter paper disc were used. MIC was defined as the lowest concentration that inhibited the visible bacterial growth (NCCLS., 2005). A negative control was also included in the test using a filter paper saturated with distilled water. The experiments were repeated at least triple.

2.5. Statistical analysis

Data were subjected to analysis of variance where significant differences existed, GLM procedure was used to test the significant concentration of S. platensis water extracts against tested strains and its minimum inhibitory concentration. These tests followed by using Duncan's test at 0.05 probability level (Duncan, 1955) to compare the significant differences in the mean numbers using SPSS Package.

3. RESULT AND DISCUSSION

Twelve traditional food products were used in the present study as source for isolation of pathogenic bacteria. Thirty five of pure single colonies were preliminary characterized by some physiological and biochemical tests according to the criteria of Bergey's Manual of Determinative Bacteriology (Holt et al., 2000). Twenty isolates showed differences in their morphological and biochemical characters were selected for further work. These isolates were named from M1 to M20 and subjected for further molecular identification. All 20 isolates were divided into three groups, Gram-positive (G+ve) bacilli, (G+ve) cocci and Gram-negative (G-ve) short rod. Twenty strains (M01-M20) were identified by determine and analysis of the partial sequence of 16S rRNA gene. The species were initially determined by the BLAST program on NCBI (http: //www.ncbi.nim. nih.gov/) based on the 16S rRNA sequences of type strains. The identity and coverage Percentage were presented in Table 1. The isolate M18 showed 100% identity and coverage with type strain Escherichia coli strain mohi KC013977. The identity percentage of the other isolates were 99%, except the isolates, M07, which recorded identity percentage of 92% with Bacillus subtilis strain p29-D09 (JQ35773) ; M09, which recorded percentage of 85% with Bacillus circulans strain BP9_5B (JN644554) ; M10, which recorded percentage of 90% with Bacillus subtilis strain M50 (JX102496) ; M12, which recorded percentage of 87% with Staphylococcus aureus strain 518F (VITSV4), and M13, which recorded percentage of 92 % with Staphylococcus aureus strain ET-1 (JX163860) . Therefore these isolates were considered as new species and named: Bacillus sp. Strain, M07; M09&M10 and Staphylococcus sp. Strain, M12 and M13. Similar cases were reported and discussed previously by Drancourt et al. (2000). They stated that 99% identity in 16S rRNA gene sequence was the criterion used to identify an isolate to the species level. A 97 to 99% identity in 16S rRNA gene sequence was the criterion used to identify an organism at the genus level, and <97% identity in 16S rRNA gene sequence was the criterion used to define a potentially new bacterial species. The phylogenetic tree was constructed using Lactobacillus paraplantarum(NR- 025447) as out of group element. The resulted phylogenetic tree (Fig.1) was composed mainly of four clusters (Cluster I – IV)

Cluster:I composed of twosub-clusters, (A) that contained Bacillus subtilis strain (M05) and three Bacillus sp strains,(M07, M09 and M10) with the type strain jQ835773. (B) Bacillus subtilis group that included, (M02, M04 and M06 with the type strain, KC443103.

Cluster:II composed of one sub-cluster (c) contained Bacillus group that included four Bacillus circulans isolates M08, M01, M11 and M03, at high similarity (98-100%) with the reference strain, JN644554.

Cluster : III composed of one sub-cluster (D) that included three Staphylococcs aures (M14, M15, and M16), at high identity percentage (99%) with the reference strain HE579073 in addition to two strains, Staphylococcs sp. M12&M13 with 87-92% identity respectively .

Cluster: IV .This cluster contained four Escherichia coli isolates; M17, M18, M19, and M20 that share high similarity (99-100%) with type strain KC013977 located in the same sub-cluster (E).

After the molecular identification, it can be stated that the isolates recovered from traded diets in restaurants are :Bacillus Subtilis (4 strains), Bacillus SP. (3 strains), Bacillus circulans (4 strains), Staphylococcus aureus (3 strains), Staphylococcus SP. (2 strains), and Escherichia coli (4 strains) Bacillus circulans isolated from rice , meat and chicken samples , while Ithas been isolated previously from different rice products (Fangio et al., 2010; Kim et al., 1998). In addition, Most of Bacillus subtilis strains were isolated from soup samples. Staphylococcus aureus was occurred in burger and shawerma chicken. These results are in agreement with Previous studies indicated that, Staphylococcus aureus has been isolated from chicken sandwish and chocolate (Iyer and Kumosani, 2010). Half isolates of Escherichia coli were isolated from unprocessed food. Same finding has been previously demonstrated by Yossa et al. (2010). They reported that most of consumption of refrigerated ready – to- eat, fresh cut fruits and vegetables, often eaten with minimal processing, were a potential source of Escherichia coli . On the other hand, Sospedra et al. (2012) stated that Escherichia coli Staphylococcus aureus were also found in several vegetable dishes, E. coli was detected in 6.6% and 0.7 % of lettuce samples. Salad ingredients were eaten fresh vegetables without cooking processes and lettuce was done of the most contaminated sample.

Antibacterial activity of S. platensis water extract of different concentrations was determined by paper disc diffusion method and the results were summarized in Table 1.All strains of Escherichia coli and Bacillus circulans showed inhibition by all concentration of S. platensis water extracts. Staphylococcus aureus strains (2 out of 5) exhibited inhibition by1 and 10 mg/ml of S. platensis, extract, while the same concentrations showed no activity against Bacillus subtilis strains. On the other hand, highest inhibition activity was shown against Bacillus circulans (M08) where inhibition zone measured 13 mm when using100mg/mlS. Platensis water extract followed by Bacillus circulans M11. Staphylococcus aureusM12-15 showed the lowest inhibitory activity (inhibition zone measured 4 mm) by the same mentioned concentration of S. platensis.The inhibitory effect of S. platensis Conc. (100mg and 50mg/ml) against twenty bacterial strains were shown in Fig.1. The present results were in harmony with those obtained by Xalxo et al. (2013) where they found that hot water extract of S. platensishad antibacterial effect against Salmonella, Shigella, Klebsiella, Streptococcus. Moreover, the zone of inhibition against tested bacteria was scored between (9-10mm). On the other hand, Singh and Rajini (2004) found that hot water extract, crude and cell free polysaccharide did not exhibit antimicrobial property. On comparing the zone of inhibition exhibited by all extracts methods of S. platensis it was concluded that the zone exhibited by the hot water extract was wider than that of other methods. Many researchers reported that organic solvents always provides a higher efficiency in extracting compounds for antimicrobial activities compared to water based methods. Also, the purified antimicrobial compounds produced byS. platensis by using thin layer chromatography was more active against Gram positive, Gram negative bacteria (El-Sheekh et al., 2014). The antimicrobial activity of microalgae could be explained by the presence of cyclic peptides, alkaloids and lipopolysaccharides. This activity may be due to the toxins produced by its cells like a number of blue green algae that produce toxins which have potential pharmaceutical applications (Katircioglu et al., 2006). Statistical analysis revealed that there are significant differences between the two concentrations (50mg and 100mg/ml) of S. platensis extract against the tested strains, where F value= 632.18 and 104.3 respectively. Moreover, the highest significant concentration of S. platensis extract (100mg/ml) was exhibited againststrains M08 and M11respectively. Meanwhile, 50 mg /ml S. platensis extract scored significant effect against five strains (M04, M05, M06, M07, M011); the other two low concentrations (1 and 10 mg/ml) seem to be less effective against tested strains.

Minimum inhibitory concentration (MIC) occurred by S. platensis water extract against tested microorganisms are listed in table (1).S. platensis showed substantial inhibitory effect on all tested bacterial strains.S. platensis was most effective against Escherichia colistrains and Bacillus circulans with low MIC value of 1µl (100mg /ml Conc.). In contrast, the highest MIC value of S. platensis was determined at 50µl (100mg /ml Conc.) for Staphylococcus aureus and Bacillus subtilis. These results are in agreement with (El- Sheekh et al., 2014) who stated that the test microorganisms differ significantly in relation to their susceptibility toS. Plantensis antimicrobial substances, Candida albicans was the most sensitive microorganism (MIC µ50g ml^{-1}); this may be attributed to the fact that the cell wall in Gram positive consist of a single layer, whereas Gram negative bacteria cell wall is multilayered structure bounded by outer cell membrane (Devi et al., 2011). On the other hand, some bacterial species did not respond to extracts of S. platensiswhereas the purified fractions showed broad–spectrum activity against different test organisms, this might be due to masking of antibacterial activity by the presence of some inhibitory compounds in the extract as observed by Sastry and Rao (1994). Statistical analysis of MIC of tested species by S. platensiswater extractrecorded highly significant differences, where F value= 352.3, on the other hand no significantdifference was found between MIC values with respect to Bacillus subtilisstrains. The highest significant MIC (1µl) at 100mg /ml Conc. of S. platensis was shown against Bacillus circulans, Escherichia coli and 2 out of 5 strains of Staphylococcus aureus.

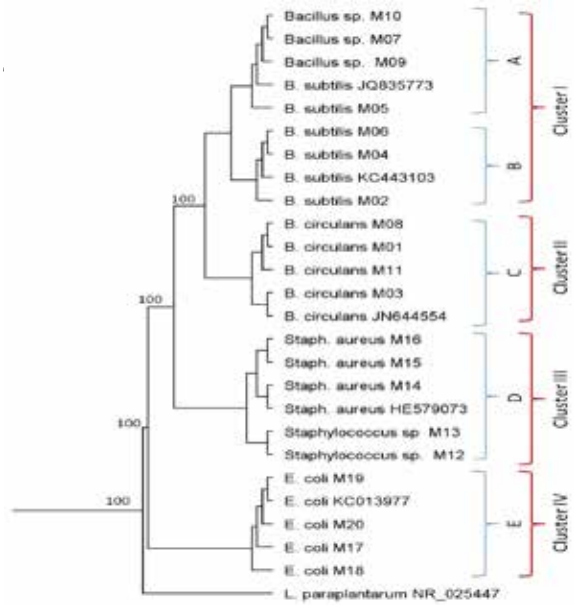
4. CONCLUSION AND RECOMMENDATIONS

Increase in bacterial infections intensified the search for new, safer, and more efficacious agents to combat serious bacterial infections. Results obtained revealed that water extracts of S. platensis showed better antibacterial activity against the pathogens used; further researches are recommended to identify and purify natural bioactive products from S. platensis as antimicrobial compounds, this will provide a new opportunity for the application of this cyanobacterium as a perfect probiotic, neutraceutical and in producing natural antioxidant food supplement or added to food and beverage products to prevent some chronic diseases where free radicals are involved.

Table 1.Identity and coverage percentage according to the obtained 16S rRNA sequence.

Isolate No.	Name and Accession No. of the most related strain in NCBI GeneBank		% Identity	% Coverage	Suggested Name of the isolates obtained in this work
M01	Bacillus circulans strain BP9_5B	JN644554.1	99	100	Bacillus circulans s M01
M 02	Bacillus subtilis strain VRC08	JX082288	99	100	Bacillus subtilis M02
M03	Bacillus circulans strain BP9_5B	JN644554	99	100	Bacillus circulans M03
M04	Bacillus subtilis strain: GS1	AB773829	100	99	Bacillus subtilis, M04
M05	Bacillus subtilis strain Sua-BAC018	EU870513	97	100	Bacillus subtilis strain M05
M06	Bacillus subtilis strain BAB-2438	KC443093	100	99	Bacillus subtilis strain M06
M07	Bacillus subtilis strain p29_D09	JQ835773	92	100	Bacillus sp . M07
M08	Bacillus circulans strain BP9_5B	JN644554	99	99	Bacillus circulansstrain M08
M09	Bacillus circulansstrain BP9_5B	JN644554	85	100	Bacillus sp. M09
M10	Bacillus subtilis strain M50	JX102497	90	100	Bacillus sp .M10
M11	Bacillus circulans strain BP9_5B	JN644554	98	100	Bacillus circulansstrain M11
M12	Staphylococcus aureus strain 518F	VITSV4	87	58	Staphylococcus sp . M12
M13	Staphylococcus aureus strain ET-1	JX163860	92	99	Staphylococcus sp. M13
M14	Staphylococcus aureus subsp. aureus ST228	HE579073	99	99	Staphylococcus aureus subsp. aureus M14
M15	Staphylococcus aureus subsp. aureus ST228	HE579073	99	99	Staphylococcus aureus subsp. aureus M15
M16	Staphylococcus aureus subsp. aureus ST228	HE579073	99	100	Staphylococcus aureus subsp. aureus M16
M17	Escherichia coli strain moh1	KC013977	99	98	Escherichia coli M17
M18	Escherichia coli strain moh1	KC013977.1	100	100	Escherichia coli strain M18
M19	Escherichia coli strain moh1	KC013977	99	100	Escherichia coli M19
M20	Escherichia coli strain moh1	KC013977	99	100	Escherichia coli M20

Fig 1. Neighbor- joining phylogenetic tree based on 16s rRNA sequence using Lactobacillus paraplantarum (NR-025447.) as out of group. The tree showing the phylogenetic placement of the strains (M01 – M20) Isolated from traded diets in restaurants at Taif city.



ulinaplatensiswater extracts against test organisms.

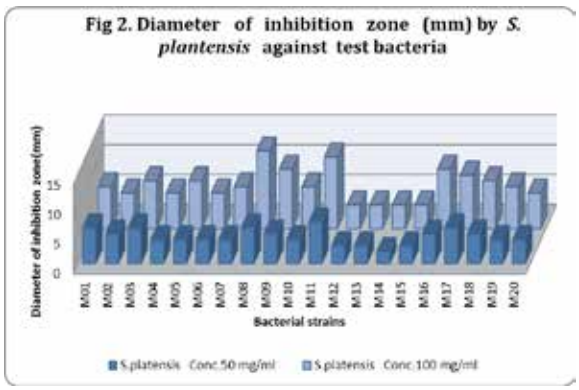
ND: not detected; diameter of disc: 2 mm. Conc; Concentration mg/ml *: mg of Spirulinaplantensisdry weight per ml distillated water; values represent the mean inhibition zones of three experiments.* the same letters vertically are not significant at p≤0.05 where Duncan's multiple range tests by using LSD test).

Table 3. Minimum inhibitory concentration of Spirulinaplatensiswater extract against tested strains.

Microbial strains	**µl/ disc***
Bacillus circulans **M01**	**1^{e}**
Bacillus subtilis **M02**	**10^{b}**
Bacillus circulans **M03**	**1^{e}**
Bacillus subtilis **M04**	**50^{a}**
Bacillus subtilis **M05**	**50^{a}**
*Bacillus subtilis***M06**	**50^{a}**
*Bacillus sp***M07**	**50^{a}**
*Bacillus circulans***M08**	**1^{e}**
*Bacillus sp***M09**	**1^{e}**
Bacillus subtilis **M10**	**50^{a}**
*Bacillus circulans***M11**	**1^{e}**
Staphyloccocussp M12	**50^{a}**
Staphylococcus sp **M13**	**50^{a}**
Staphylococcus aureus subsp. aureus **M14**	**50^{a}**
Staphylococcus aureus subsp. aureus **M15**	**1^{e}**
Staphylococcus aureus subsp. aureus **M16**	**1^{e}**
*Escherichia coli***M17**	**1^{e}**
Escherichia coli **M18**	**1^{e}**
*Escherichia coli***M19**	**1^{e}**
Escherichia coli **M20**	**1^{e}**
L.S.D	**2.30**
F value	**3523**

Microbial strains	Diameter of inhibition zone (mm) by S.platensis(Conc.mg/ml)			
	1	10	50	100
Bacillus circulansM01	2	4	6^{b}	7^{f}
Bacillus subtilis M02	ND	ND	5^{c}	6^{g}
Bacillus circulansM03	2	4	6^{b}	8^{e}
Bacillus subtilisM04	ND	ND	4^{a}	6^{g}
Bacillus subtilisM05	ND	ND	4^{a}	8^{e}
Bacillus subtilisM06	ND	ND	4^{a}	6^{c}
Bacillus subtilisM07	ND	ND	4^{a}	7^{f}
Bacillus circulansM08	2	3	6^{b}	13^{a}
Bacillus circulansM09	2	3.6	5^{c}	10^{c}
Bacillus subtilisM10	ND	ND	4^{d}	7^{f}
Bacillus circulansM11	3	5	7^{a}	12^{b}
Staphylococcus aureuM12	ND	ND	3^{e}	4^{h}
Staphylococcus aureuM13	ND	ND	3^{e}	4^{h}
Staphylococcus aureus subsp. aureus M14	ND	ND	2^{f}	4^{h}
Staphylococcus aureus subsp. aureusM15	3	3	3^{e}	4^{h}
Staphylococcus aureus subsp. aureus M16	2	4	5^{c}	10^{c}
Escherichia coli M17	2	4	6^{b}	9^{d}
Escherichia coli strain M18	2	4	5^{c}	8^{e}
Escherichia coli M19	2	3	4^{d}	7^{f}
Escherichia coli M20	1	2	4^{d}	6^{g}
L.S.D			0.278	0.58
F value			104.3	632.18

Value represent the mean inhibition zones of three experiments;µl/ disc* from 100mg ofS. Plantensisdry weight per mldistillated water. *The same letters vertically are not significant at p≤0.05 where Duncan's multiple range tests by using LSD test).



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