The Anti-Microbial Properties of *Triticum aestivum* (Wheat Grass) Extract

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Abstract: Wheat grass, one of the members of *Poaceae* family, has been considered for very efficient therapeutic drugs. Current study was aimed at evaluation of antimicrobial properties of wheat grass extracts. The 7$^{th.}$, 14$^{th.}$, and 21$^{st.}$ day wheat grass extracts of five different solvents (water, ethanol, methanol, ethyl acetate and hexane) were assayed for antimicrobial activity using turbidity tests. All these extracts showed antibacterial activity against seven food borne pathogens. Amongst them hexane extracts from 7th day old wheat grass showed maximum antibacterial activity especially more against *Yersinia enterocolitica* and *Listeria monocytogenes*. The HPLC purified extract was observed to create pores on the cell wall of the bacterial cells as observed under Scanning Electron Microscope and also influenced flattening and shrinkage of bacterial cells indicating probable effect on the membrane of the pathogenic bacteria.

Keywords: *Triticum aestivum*, Antimicrobial activity, Solvent extraction, Bioactive molecule, SEM.

INTRODUCTION

Herbal or 'alternative' medicine is gaining popularity and scientific research about wheatgrass as a "functional food" is becoming more available and popular. Wheat grass, *Triticum aestivum* L has a long history and is widely used as a health food supplement. It is found to be used as a treatment for minor ailments and serious life threatening issues, and also as a preventative dietary supplement. The use of chlorophyll in trials offers a broad view of the potential for wheat grass or wheatgrass derivatives. Another interesting quality is the antioxidant potential of wheat grass extract in particular its superoxide dismutase (SOD) content. This enzyme has gained much more attention in recent years with regard to its ability to inhibit cell mutation [1]. The use of wheat grass, and particularly its fresh juice became popular again in the 1970s, when Ann Wigmore wrote '*The Wheat grass Book'.* Even though the book itself is homage to the applications of wheat grass, it does not make reference to whether the chosen applications offer valid scientific data or not. Ann Wigmore also established the famous Hippocrates Centre treating thousands of clients with herbal grasses and wheatgrass juice. A study found in the book 'Wheat grass Natures finest medicine' by Steve Meyerowitz published in 1983, describes the treatment of active distal ulcerative colitis and the effect of wheat grass on its activity when taken as a dietary supplement [2]. Ashok *et al.* (2011) has described the phytochemical and pharmacological screening of *T. aestivum* where it has been shown to be a rich source of Vitamins A, C, E and B complex, including B12 [3]. It contains a multitude of minerals like calcium, phosphorus, magnesium, alkaline earth metals, potassium, zinc, boron, and molybdenum. Other compounds which make this grass therapeutically effective are the indole compounds, choline and laetrile (amygdalin) [4]. Glycoside molecules, which are also a powerful antioxidant, have also been isolated as having potential to inhibit DNA oxidative damage *in-vitro* [5]. Further, the medicinal properties of the wheat grass have been shown to change at different growing conditions [6]. Crude phyto-drugs may be less efficient than modern medicines, but they are relatively free from side effects. Thus, there is an increasing need for efficient, cost-effective, safer medicinal agents with little or no side effects [3]. The majority of information available is in the form of anecdotal literature, usually published by a company selling a wheatgrass product. However, not enough in-depth controlled clinical trials have been conducted to study the therapeutic effect of wheatgrass [7]. Another common claim amongst the anecdotal literature is that the wheat grass juice has antimicrobial properties. There appears to be a lack of published scientific data on this topic, thereby highlighting an area for continued research. Pallavi *et al*. (2011) tested wheatgrass extracts against the Gram-positive bacteria; *Staphylococcus aureus, Bacillus subtilis* and Gram-negative *Escherichia coli*; using Amoxicillin as standard. Certain extracts exhibited considerable activity against *Bacillus subtilis* and moderate activity against *Staphylococcus aureus and Escherichia coli* [8]. Ashok (2011) also reported antibacterial activity against *Escherichia coli; Pseudomonas aeruginosa* and *Saphylococcus aureus*. Antifungal activity was also reported against *Candida*

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albicans [3]. Das *et al*. (2012) found 80% acetone extracts of wheatgrass were effective against five food-borne microorganisms, including the fungus *Aspergillus niger*, a common contaminant of food [9].

Due to bacterial expression of resistance to antibiotics, the development of new antiseptics and antimicrobial agents are of growing interest [10]. Plants contain thousands of constituents and are valuable sources of new and biologically active molecules having antimicrobial properties [11]. A wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, have been found to have antimicrobial properties *in vitro* [12]. These natural products are of concern as a source of safer and/or more effective alternatives to synthetically produced antimicrobial agents [13]. Wheat grass extract has a high content of bioflavonoids which may add towards antimicrobial effects [4].

Hence, it was proposed to work on the beneficial effects of wheat grass extract. In this communication we present bactericidal / bacteriostatic activities of wheat grass extracts on a few food borne pathogens.

MATERIALS AND METHODS

Growing and Harvesting of Wheatgrass

Wheat, *Triticum aestivum* was purchased from the local market of Mysore, India. Wheat was sown in plots of 48'' X 12'' X 12'' and watered daily. Wheat grass was harvested on 7^{th}, 14^{th} and 21^{st} day

Microorganisms

Food borne bacterial organisms selected were Staphylococcus aureus MTCC (Microbial Type Culture Collection) 96, Salmonella parathyphi MTCC 3231, Listeria monocytogenes MTCC 1143, Streptococcus pneumoniae MTCC 655, Yersinia enterocolitica MTCC 859, Bacillus cereus MTCC 1272 and Escherichia coli MTCC 729. They were purchased from IMTECH (Institute of Microbial Technology), Chandigarh, India. All test microorganisms were maintained on Nutrient agar slants at 4°C ±1°C. Sub culturing was done once in 15days.

Extraction of Wheat Grass Bioactive Compounds

Wheat grass harvested on day 7, 14 and 21 were homogenised with different solvents - water, methanol, ethanol, hexane and ethyl acetate using electronic stirrer. The samples were stirred for 24h at ambient temperature (26 – 28 °C) followed by centrifuging at 5000 rpm (Rotations per minute) for 5 minutes. Then supernatant was carefully removed. The solvent was evaporated under vacuum at 50 °C. Each dried extract was resuspended in DMSO (Dimethyl Sulfoxide) and sterilised by membrane filtration using 0.0.45µm pore size sterile filters. Extracts were preserved at 4 °C±1 °C until use.

Antimicrobial Activity Analysis

Pre-screening for anti-microbial activity was done using the agar well diffusion method. A broth suspension of each pathogen under study was obtained by inoculating a loop of each bacterium to 3ml of nutrient Broth. The inoculated tubes were incubated in a bench-top orbital shaking incubator at 150 rpm, 37 °C for 24 hours.

Petri-plates containing nutrient agar medium were seeded with 0.1 ml of 24 hr culture of bacterial strains by spread plate technique. Wells were made in these agar medium using a sterile cork borer and then 100µl of the DMSO suspended wheat grass extract was added to the wells. Control wells with only DMSO were also maintained. Plates were incubated at 37 °C for 24 hours. Activity was determined by observing the formation of a zone of inhibition around each well.

Quantitative Determination of Antibacterial Activity

3mL nutrient broth was inoculated with overnight culture of respective organism. 50µL of DMSO suspension of the wheat grass extracts were added and the tubes were incubated at 37 °C for 24 hours in a shaker incubator (150rpm). Following day, the tubes were centrifuged and then the cells were washed with PBS (Phosphate-buffered Saline) and resuspended in PBS. OD (Optical Density) was measured at 600nm. Controls with only DMSO and controls without any additives were also maintained. The inhibition percentage was calculated by comparing it to that of control (presumed to be 100%).

Age of Wheat Grass and Antimicrobial Activity

3mL nutrient broth was inoculated with overnight culture of respective organism. 50µL of DMSO suspension of the wheat grass extract of 7, 14 and 21 days old wheat grass were added separately and the tubes were incubated at 37 °C for 24 hours in a shaker incubator (150rpm). Following day the tubes were centrifuged, the cells were washed with PBS and resuspended in PBS. OD was measured at 600nm.

Controls with only DMSO and controls without any additives were also maintained. The inhibition percentage was calculated by comparing it to that of control (presumed to be 100%).

Bacterial Morphology by Scanning Electron Microscopy (SEM)

The antibacterial activity of the hexane extract of wheatgrass on bacterial morphology (Gram positive and Gram negative) was evaluated by observing cells under scanning electron microscopy (SEM). The inoculated Nutrient Broth without adding crude hexane extract was used as control. The test micro-organisms were incubated in nutrient broth containing DMSO suspension of hexane extract for 24 h. The treated pathogen cultures were harvested at $16^{th.}$ and $24^{th.}$ hour time of incubation and centrifuged at 10,000 rpm for 15 min to collect the cell pellet. The cell pellet was washed twice with PBS buffer (0.01 M; pH 7.0) followed by the overnight fixing of the pellet with 2 % glutaraldehyde. Then the pellet was dehydrated in ethanol on a gradient mode (10–100 %). Upon completion of wash with 100 % ethanol, the lyophilized pellet was spread on to the metallic stubs. These metallic stubs were sprinkled with gold under vacuum and analysis was performed using a SEM (LEO 435VP, UK) attached to a video copy processor (Mitsubishi, Japan). The images were then photographed and analysed for morphological changes [14].

Determination of Minimum Inhibitory Concentration (MIC)

MIC is the lowest concentration of a test material at which no micro-organism growth occurs. Plates were prepared under aseptic conditions. A sterile 96 well micro-titre plate was labelled. 100 µL of test material in 10% (v/v) DMSO or sterile water (usually a stock concentration of 1 mg/mL for purified compounds and 10 mg/mL for crude extracts) was pipetted into the first row of the plate. To all other wells, 50 µL of nutrient broth or normal saline was added. Serial dilutions were performed using a multichannel pipette. Tips were discarded after use such that each well had 50 µL of the test material in serially descending concentrations. Using a pipette 30 µL of 3.3 × strength nutrient broth was added to each well to ensure that the final volume was of single strength of the nutrient broth. Finally, 10 µL of bacterial suspension (5×10^6 cfu/mL) of *Listeria monocytogenes* was added to each well to achieve a concentration of 5×10^5 cfu/mL. Each plate was wrapped loosely with cling film to prevent bacteria from getting dehydrated. Each plate had a set of controls: a column with a broad-spectrum antibiotic as positive control (usually ciprofloxacin in serial dilution), a column with all solutions with the exception of the test compound, and a column with all solutions with the exception of the bacterial solution adding 10 µL of nutrient broth instead [15]. Similar experiments were carried out with *Yersinia enterocolitica.* The plates were prepared in triplicates and placed in an incubator set at 37 °C for 18–24 h. Then the plates were read at 600nm for growth using Varioskan plate reader.

RESULTS

Antimicrobial Activity of Different Solvent Extracts of Wheat Grass

To find out the best extracting solvent, the wheat grass extracts from different solvents were screened, using agar well diffusion method. Clear zones were observed (Table **1**) for all pathogens studied. But *Yersinia enterocolitica, Staphylococcus aureus and Listeria monocytogenes* were observed to be most sensitive pathogens.

Table 1: Occurrence of Inhibition Zone in Agar Well Diffusion Test

Food pathogens	Extracting Solvent				
	Water	Ethanol	Methanol	Ethyl acetate	Hexane
Salmonella typhi	-	+	+	+	+
Yersinia enterocolitica	+	+	+	+	+
Bacillus cereus	+	+	+	+	+
Staphylococcus aureus	+	+	+	+	-
Streptococcus pneumonia	-	-	+	+	+
Listeria monocytogenes	+	+	+	+	+
Escherichia coli	-	+	+	+	+

(+) - zone of inhibition.
(-) - absence of zone of inhibition.

Quantitative Estimation of Antibacterial Activity

Quantitative estimation of the viable biomass after treatment with wheatgrass extracts indicated that wheat grass extracts had differential effects on each microorganism under study. Methanol extract of wheat grass had maximum inhibitory effect on *Salmonella paratyphi* (Figure **1**). With *Yersinia enterocolitica,* hexane extract gave maximum inhibition of 72.6% followed by ethyl acetate (61.4%). B*acillus cereus* was more resistant to any type of extract of wheat grass. Maximum of 20% inhibition was observed with ethyl acetate extract of wheat grass. *Staphylococcus aureus* showed 47.6 and 45% inhibition respectively with ethanol and methanol extracts of wheat grass. Ethyl acetate and hexane extracts showed no inhibition of *Staphylococcus aureus while a*queous extract of wheat grass showed only 11 % inhibition of growth. *Streptococcus pneumoniae* showed inhibition with all types of extracts. Of these, hexane extract inhibited the growth of *Streptococcus pneumoniae* by 91% followed by ethyl acetate and methanol extracts and ethanol extract showed the lowest inhibitory activity of 28%. Growth of *Listeria monocytogenes* was also inhibited by extracts of wheat grass with all solvents studied. Ethanol extract had lowest inhibition (39%) while hexane extract inhibited the growth of *Listeria monocytogenes* completely. Also Ethyl acetate, aqueous and methanol extracts showed 61.3, 49.3 and 46.6 % inhibition of growth respectively.

Age of Wheat Grass and Antimicrobial Activity

The age of the wheat grass appeared to play a major role in the presence or absence of active antimicrobial ingredient. The hexane extract from 7 days old wheat grass showed inhibitory activities against few food borne pathogens used. Highest inhibition of 82.56% was observed with *Listeria monocytogenes* followed by *Streptococcus pneumoniae* (75.69 %). No activity was observed against *Staphylococcus aureus.* Only 2.84 % inhibitory activity was observed against *Bacillus cereus* (Figure **2**). 7 days old wheat grass had antibacterial activity against *Salmonella paratyphi* while 14 days and 21 days old wheat grass had no activity against *Salmonella paratyphi.* Similarly, 7 days old wheat grass had activity against *Yersinia enterocolitica* while hexane extract of 14 days old wheat grass had no activity against *Yersinia enterocolitica*. Activity of hexane extract of 21 days old wheat grass was very low (16%). With *Streptococcus pneumoniae,* activity of hexane extract of 7 days old wheat grass was 75.69 %. The activity increased with hexane extract of 14 days

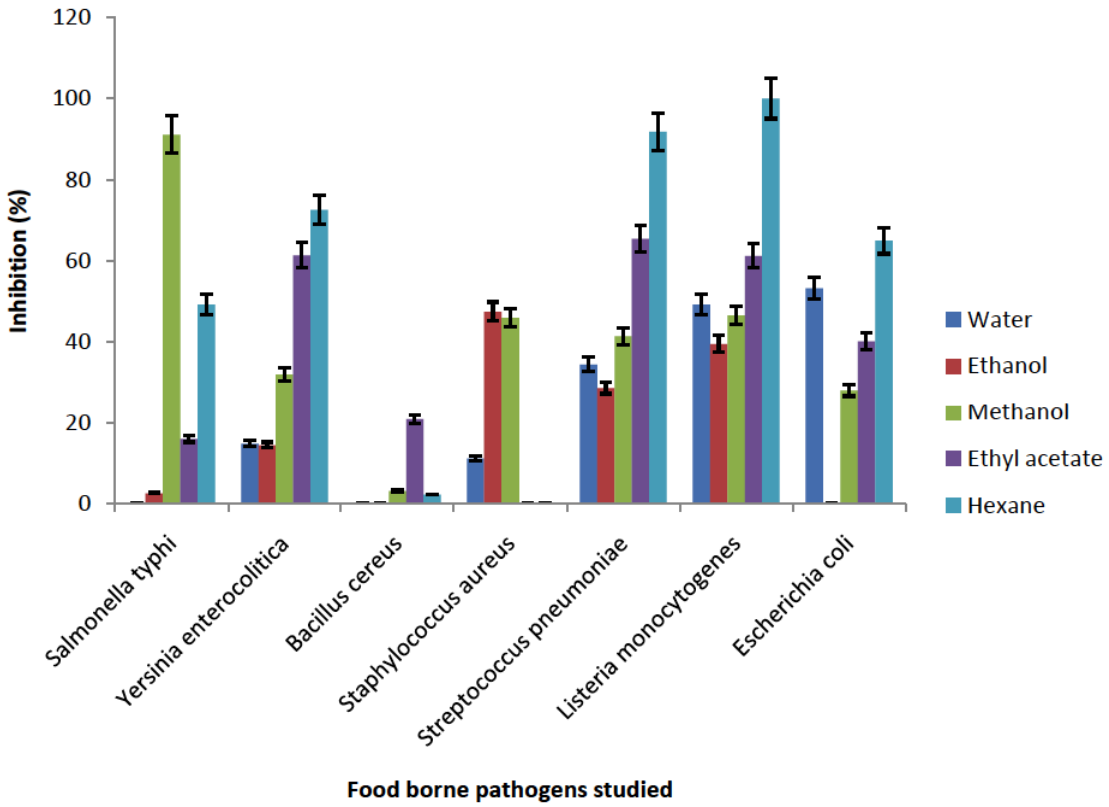

Figure 1: Quantitative estimation of antibacterial activity using different solvent extracts.

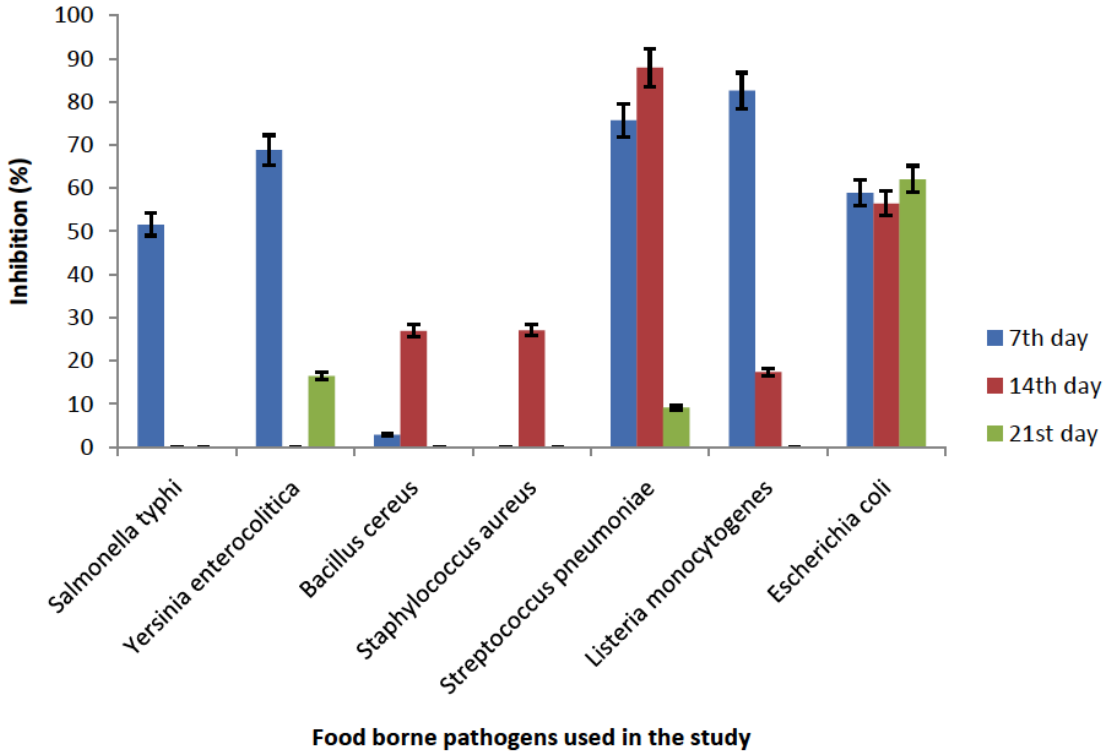

Figure 2: Quantitative estimation of antibacterial activity of 7th, 14th and 21st day hexane extracts.

old wheat grass (87.90%) and decreased with hexane extract of 21 days old wheat grass (9.14%). Hexane extract of 21 days old wheat grass had highest activity against *Escherichia coli* (62.02 %).

Bacterial Morphology Changes Observed in SEM

On comparing the surface morphology in control cells of gram positive and gram negative strains with 16th. and 24th. hour (after treatment with antimicrobial) respectively, it can be inferred that there was visible action of hexane extract on bacteria surface. When the cellular permeability gets altered, the cell dies eventually (Hartmann *et al*., 2010). Other changes in the morphology of 16th. hour sample of both gram positive and gram negative were the bifurcation of few cells (absent in control) and flattening of cells at the centre leading to dumb-bell shape formation in *Yersinia enterocolitica*. Also coagulation of cells got initialized in the 16th hour in gram positive cells compared to gram negative cells (which required 24 hr to coagulate). This also supported the fact that gram negative bacteria studied were slightly more resistant compared to gram positive bacteria. This may be due to the impermeability of bio-actives caused by LPS (Lipo-polysaccharides) in their membrane. Overall shrinkage in cell size was also observed with increasing time of exposure. The 24th. hour cells of *Yersinia enterocolitica* showed clear fragmentation of cells (Figure **3**).

Determination of MIC (Minimum Inhibitory Concentration)

The minimum inhibitory concentration (MIC) is the concentration at which an antibacterial agent experiences the complete inhibition of micro-organism growth. MICs are considered as golden standard for determining the susceptibility of microorganisms to antimicrobial compounds. In our studies, it was observed that for *Listeria monocytogenes,* MIC was 168.6µL with an IC_{50}= 84.30µL and with *Yersinia enterocolitica,* MIC was 113.14µL with an IC_{50}= 56.57µL.

DISCUSSION

The Human diet is enriched with young parts of plants (so called green foods), which can improve nutrient balance intake in natural way. Wheatgrass (*Triticum aestivum*) refers to young grass of the common wheat plant, which belongs to *Poaceae* family. This is the most commonly found herb in India. This plant is believed to have many nutritional values; it has been shown to have anti-inflammatory, antioxidant, anti-carcinogenic, immune-modulatory, laxative, astringent, diuretic, antibacterial and anti-aging properties. Its use in acidity, colitis, kidney malfunctions, atherosclerosis and swelling has been shown to be beneficial [16,17]. Wheatgrass packs a

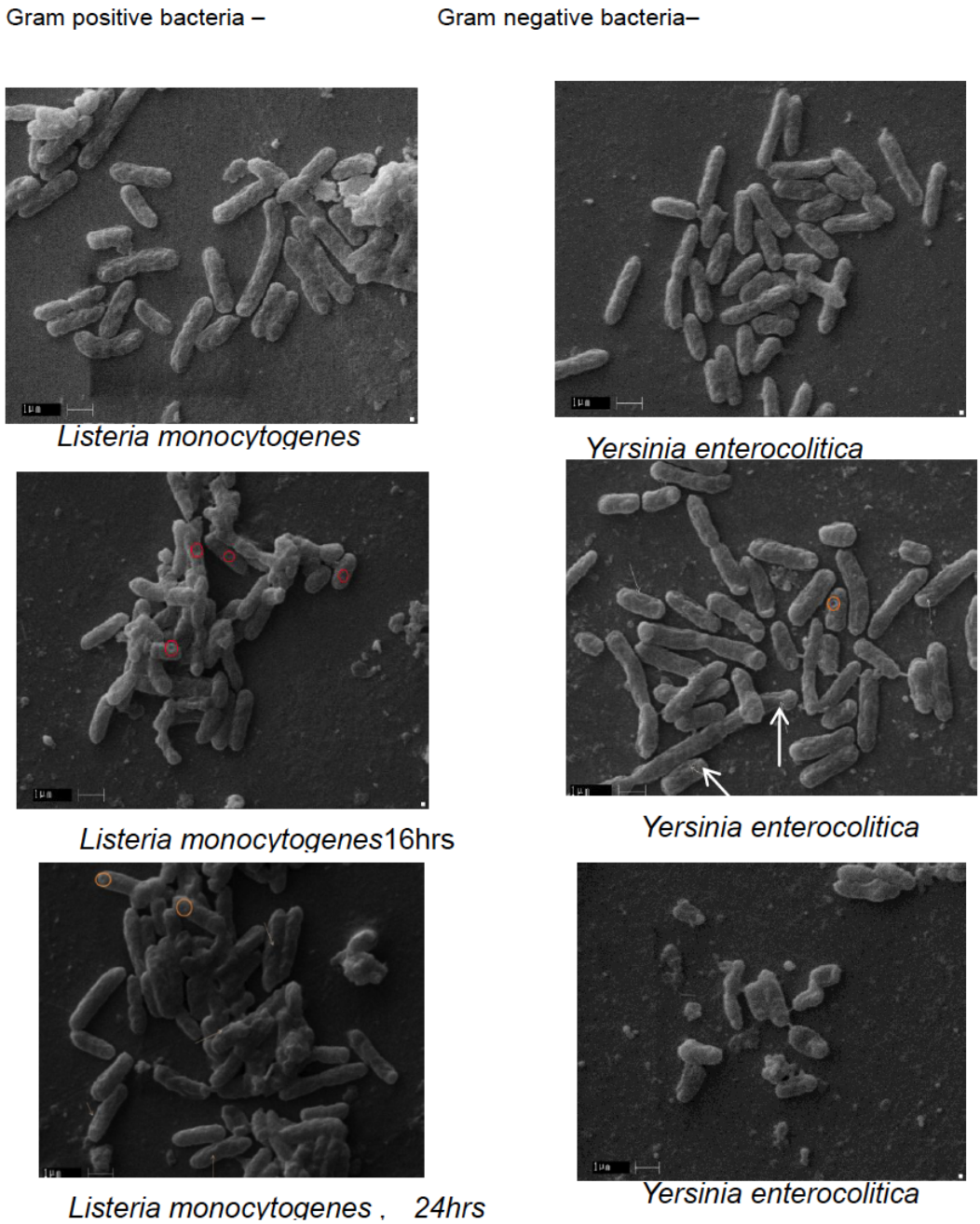

Figure 3: SEM images of gram positive(left) and gram negative(right) treated with DMSO suspension of hexane extract.

⬭ Indicates the pores formed on the surface.

→ Indicates the folding formed due to the antimicrobial bioactive present in the wheatgrass hexane crude extract.

nutritional punch, including (per 3.5 grams) 860 mg protein, 18.5 mg chlorophyll, 15 mg calcium, 38 mg lysine, 7.5 mg vitamin C and an abundance of micronutrients, such as B complex vitamins and amino acids [17]. Phytochemical constituents of wheatgrass include alkaloids, carbohydrates, saponins, gum and mucilages. Its water soluble extractive value is found to be greater than its alcohol soluble extractive value [3]. Wheat grass juice is high in vitamin K, which is a blood-clotting agent. Wheatgrass leaf extract improves the digestive system, and promotes general well-being [18-20]. It has higher nutritive value than broccoli and spinach [21]. Its high levels of enzymes and amino acids work like a natural cleanser to detoxify the liver, eliminate toxic heavy metals from the blood stream, rid the body of waste matter and slow down the aging process [22]. With these beneficial effects of wheat grass extracts, in our study we attempted to study the antimicrobial effect of wheat grass extracts. We obtained antimicrobial activity of aqueous extract of

wheat grass against some of the food borne bacterial pathogens. Pallavi *et al*. (2011) reported activity in acetone WGJ (Wheatgrass juice) extracts against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* [8]. Das *et al.* reported activity in 80% acetone extracted samples against four bacteria: *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli, Shigella flexneri* and one fungus: *Aspergillus niger* [23]. However, Desai (2005) found acetone and methanolic extracts did not show any antibacterial activity while fresh and undiluted wheatgrass juice exhibited mild antibacterial activity against *Staphylococcus aureus, Bacillus cereus, Salmonella typhimurium* and *Kleibsella pneumoneae.* It is interesting to note that in the study by Desai (2005) the efficacy of fresh WGJ declined after 2 hours. Fresh undiluted wheatgrass juice was then screened against bacteria: *Escherichia coli* NCTC 10418; *Staphylococcus aureus* NCTC 6571 and *Streptococcus mutans* NCIMB 702062. None of the extracts tested displayed any kind of antimicrobial activity against selected pathogens [24].

CONCLUSION

Inactivation of pathogenic microorganisms or microorganisms that cause food spoilage is one of the basic purposes of food preservation methods. The objective of this study was to explore the benefits of wheatgrass extract with respect to its antibacterial potential. The extracts of wheat grass were found to possess antibacterial activity against some of the major food borne pathogens used in this study. The antimicrobial activity was clearly dependent on the age of the wheat grass as well as the solvent used for extraction. Thus wheatgrass extract would be a novel antimicrobial agent. More optimisation can be carried out to obtain extract exhibiting very high antimicrobial activity. The Wheatgrass extracts can also be used as a health tonic with the additional nutritional benefits they provide. Wheatgrass extracts being natural medicine can be extremely valuable for treating various sicknesses from minor scratches and blazes to genuine infections. Plant products are of increasing interest in the search for new drugs and medicines in the treatment of diseases. I conclude by saying that there is a vast scope of research and innovations for wheat grass (*Triticum aestivum*) and its formulations as highly effective antimicrobial agents.

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