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INVESTIGATING THE MINIMUM INHIBITORY CONCENTRATION (MIC) OF THE *HIBISCUS ROSA-SINENSIS* WINE AGAINST COMMON FOOD BORNE PATHOGENS

***Snehlata Tiwari[1] and Sangeeta Shukla[2]**

[1]Research Scholar, Warner School of Food and Dairy Technology, Sam Higginbottom Institute of Agriculture Technology and Sciences, Allahabad, India.

[2]Assistant Professor, Warner School of Food and Dairy Technology, Sam Higginbottom Institute of Agriculture Technology and Sciences, Allahabad India.

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***Corresponding Author**
Snehlata Tiwari
Research Scholar, Warner School of Food and Dairy Technology, Sam Higginbottom Institute of Agriculture Technology and Sciences, Allahabad, India.

ABSTRACT

The potential antimicrobial activity of *Hibiscus rosa-sinensis* wine against food borne pathogens was studied using the agar well diffusion method. The infections risk related to pathogenic germs increases at the present time considering the increased resistance which certain microbes acquire, whose usual antibiotics are ineffective to treat the infectious disease. Our results are of great practical importance as the prepared herbal wines besides being a tasteful addition to food might also prove to be a health drink with antimicrobial potential against a variety of food-borne pathogens. The aim of this study was to determine antimicrobial effect of the *Hibiscus rosa-sinensis* wine prepared from different yeast strains of *Saccharomyces cerevisiae* i.e. MTCC 178, MTCC 180, MTCC 786, against common food borne pathogens viz. *Bacillus subtilis*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*. In this experimental study, after collecting Hibiscus flowers, the extraction was kept for fermentation to prepare Herbal wine, after antimicrobial effect of the wine evaluated by Minimum Inhibitory Concentration (MIC) determined by using a dilution method. Statistical analysis was carried out by analysis of variance (ANOVA). The results shows that wine prepared from *strains* MTCC 178, MTCC 180, MTCC 786 were quite effective in 2000 µg/ml concentration on *Bacillus subtilis*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*. wine prepared from *strains* MTCC 180, MTCC 786 *has effective impact* was 16 and 32 mg/ml respectively. Antimicrobial study against food

borne pathogens showed growth inhibitory effect in the range of 11-16 mm. Almost all the samples of Hibiscus wine showed zone of inhibition against food borne pathogens, While The Hibiscus wine prepared from MTCC 178 presented the more effective impact on the growth of food borne pathogens.

KEYWORDS: *Hibiscus rosa-sinensis,* MTCC 178, MTCC 180, MTCC 786 *Bacillus subtilis, Salmonella typhimurium, Staphylococcus aureus, Escherichia coli,* antimicrobial activity.

INTRODUCTION

The increasing antimicrobial resistance of pathogens isolated from humans and animals, combined with the increasing awareness of the consumers on chemical substances used as food preservatives, necessitates research for more efficient antimicrobials with fewer side-effects on human health. Recently, the antimicrobial effects of various plant extracts against certain pathogens have been reported by a number of researchers. Particularly, polyphenols of plant origin have been reported to have a variety of biological effects, including anti-oxidant, anti-carcinogenic, anti-inflammatory and anti-microbial activities. Specifically some phenolic compounds such as resveratrol, hydroxytyrosol, quercetin and a number of phenolic acids have been reported to inhibit various pathogenic microorganisms (*9–12*). Also, there are recent studies reporting the antimicrobial activities of wines and wine extracts against various pathogens.

The *Hibiscus rosa-sinensis* belongs to the family Malvaceae. Traditionally flower can be used as anti asthamatic agents. many chemical constitute such as cyanidin, quercitin, hentriacontane, calcium oxalate, thiamin, riboflavin, niacin, and antimicrobial substances are present in this flower **(**Zhao J, 2010**).** The petal have some protective mechanism against microbial attack in most of the parts. The *H. rosa-sinensis* flower petal of large number of plant species growing in the vicinity of our environment were screened for their antimicrobial activity. the antioxidant properties of flavonoids, polyphenolic Compounds and anthocyanins contained in the flower can prevent the oxidation of Low-Density Lipoproteins (LDL), which is associated with the disease.

In vitro studies indicate that, for a given ethanol concentration, wine has a more potent antibacterial activity than other alcoholic beverages. This potency has been partly attributed to the combination of ethanol and organic acids (tartaric, malic, lactic and acetic) (Just &

Daeschel, 2003; Weisse, Eberly, & Person, 1995). Malic and tartaric acids are the most abundant organic acids in wine, and their antimicrobial effects are wellknown, especially in low pH conditions, such as those found in wines (Hsiao & Siebert, 1999; Ricke, 2003).

Wines have a potent antimicrobial property against different food borne pathogens which has been demonstrated under various experiments. Various studies described the antimicrobial properties of wine against different relevant food borne pathogens. Some studies indicated that the strength of antimicrobial property of wine is attributed to different components of wines and a better antimicrobial proficiency associated with red wine than white wine because of high level of phenolic compounds in red wine (Chanyalew and Wiriya, 2013). However, there are no reports so far on the antimicrobial activity in Hibiscus wine. Therefore, this study was aimed at checking the antimicrobial activities in the Hibiscus wine samples prepared with different concentrations of Hibiscus petals using different strains of *Saccharomyces cerevisiae*. The aim of this study was to determine antimicrobial effect of the Herbal wine prepared from *Hibiscus rosa-sinensis* on *Bacillus subtilis, Salmonella typhimurium, Staphylococcus aureus, Escherichia coli.*

MATERIALS AND METHODS

The material and methods were adopted during this investigation are given below -

- *Hibiscus rosa-sinensis* flowers were collected from Agronomy department of SHIATS, Allahabad.
- Sugar, KMS, Di-ammonium phosphate were purchased from local market of Allahabad
- Different types of *Saccharomyces cerevisiae* strains MTCC 786, MTCC 178, MTCC 180 were collected from IMTECH, Chandigarh, India.

Preparation of glass wares

All the glass wares were washed thoroughly with detergent or sulfuric acid. Glass wares after drying were wrapped with paper and kept in an electric hot air oven for sterilization at 160^0C-180^0C for 3-4 hours and media sterilized in autoclave at 15 lb pound pressure at 121^0C and opened only in laminar flow. Laminar flow was sterilized with the help of violet lamp for 30 minutes at room temperature. All necessary precaution was taken to avoid the contamination from outside.

Preparation of Media

All the cultures were grown at 30^0c under agitation(150 rpm) for 24h in yeast extract peptone dextrose broth (YEPD) medium and adjusted to pH 5.0. All the strains were sub-cultured on YEPD agar slants at fortnight intervals and stored in refrigerator at $4\pm^0$C, until further use. for muller hinton broth and agar, Beef Extract Peptone, Hydrolysate Acid of Casein, Starch were heated in one litre of distilled water. Then boiled, agar was added to the above mixture and stirred constantly. pH was adjusted to 7.2 by using 1.0 N HCL and NaOH drop wise. The media in the conical flask was plugged with cotton and sterilized in autoclave at 15 lbs pressure for 15-20 minutes. When the media was cooled, it was stored in the refrigerator and heated whenever required. The turbidity was measured by adjusting to 0.5 Mac Far land standards.

Maintenance of yeast culture

Three yeast strains namely *Saccharomyces cerevisiae* MTCC178, *S.cerevisiae* MTCC 180, *S.cerevisiae* MTCC 786, were procured from institute of Microbial Type Culture Collection, IMTECH, Chandigarh, India.

Bacterial strains and growth conditions

Bacterial strains used in the study were *Escherichia coli*, *Salmonella typhimurium*, *Bacillus subtilis* and *Staphylococcus aureus* obtained from the institute of Microbial Type Culture Collection, IMTECH, Chandigarh, India. All cultures were propagated in Muller Hinton broth. The culture isolates were all aerobically incubated at 37°C for 24.

Preparation of Must

The Hibiscus petals were manually sorted and washed in cold water to remove dirt. About 80 gm of the hibiscus petals powder boiled in 1000 ml of water at a starting temperature of 80^0C for 10 minutes.

Fermentation of Must

The extracts obtained were then ameliorated or enhanced, using of table sugar to raise the sugar level to 24° Brix, which is in the range of sugar levels tolerable by most yeast for alcohol production (Bisson, 2001). Brewer's yeast *Saccharomyces cerevisiae* was used in the fermentation process. An amount of 2 ml of the yeast was added for each 1 litre of must. Sulfur dioxide was added to the must in the form of sodium metabisulphite at a concentration of 0.4 g/l to stabilize the must. The must were poured into three different sterile plastic

fermenting containers and seeded aseptically with 2 ml yeast culture and stirred for even distribution of the yeast. A vent was made on the lid and stuffed with sterile cotton wool to allow for the exchange of gases. The musts were subjected to primary fermentation at 28 ± 2°C for eight days to produce the fermented must. The yeasts were filtered out to obtain the young wine.

Determination of Minimum inhibitory concentrations (MIC)

MIC was determined according to agar dilution method. Various concentrations of extract were prepared in 10 cm experimental tubes containing Mueller Hinton Broth for fungi and 10 cm experimental tubes containing Mueller Hinton Broth for bacteria. Each tube contains 9 ml of Mueller Hinton for bacteria were sterilized by autoclaving. On cooling, 1 ml of each extract concentration were added to each tube, to make the final concentrations of 2, 4, 8, 16, 32, 64, mg/ml. The mixture of Muller Hilton and extract was poured into plates aseptically in a laminar flow cabinet. On solidification of the agar medium, 2 µl of adjusted spore suspension were added to each plate by micropipette and incubated at 37C° for bacteria. The MIC was regarded as the lowest concentration of the extract that did not show any visible growth after 24 hours of incubation. After incubation time, the diameter of free zone was measured exactly by using a ruler in millimeters. All experiments were performed with 3 replicates.

Zone of inhibition by Agar well diffusion assay

An agar well diffusion assay was performed to determine if the organisms were susceptible to Hibiscus Wine. Isolates of *Escherichia coli, Salmonella, Bacillus subtilis, Staphylococcus aureus* were from the culture collection of the MTCC, Chandigarh. The Wells were aseptically cut out of each agar plate with a sterile metal cork borer of 6 mm diameter. The three wells in each plate were filled with 100 µl of different percentages of each Hibiscus wine to be tested. The plates were accordingly incubated at 37°C and observed after 24 h. The diameter of inhibition zones surrounding each well was measured. The experiment was repeated. Concentrations of the selected wines that gave the most inhibitory effects on the tested pathogens were analyzed as described in Result & Discussion.

Statistical analysis

All the assays were carried out in triplicates. The experimental results were expressed as mean ± standard deviation. The data were analyzed using one way analysis of variance

(ANOVA). Differences were determined using Fisher's Protected Least Significant Difference (LSD) with $P \leq 0.05$.

RESULTS AND DISCUSSION

Almost all the samples showed antimicrobial activity. It may be because of the presence of secondary metabolites like polyphenol, flavonoids etc, by the yeast from Hibiscus petals after. Wine MTCC 786 shows a good antimicrobial activity against *Salmonella typhimurium Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli.*

The results of the antimicrobial effects of Hibiscus Wine MTCC 178, MTCC180 and MTCC 786 by the agar diffusion method are presented in following tables. The results show that Hibiscus Wine MTCC 178 at concentrations (2,4 and 8 mg/ml) in table-1 had less significant antimicrobial effect on *Salmonella typhimurium Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli.* and it is not able to prevent the growth of bacteria on culture ($p<0.05$). However, (16, 32 and 64 mg/ml) Hibiscus Wine MTCC 178 concentrations, have significant antimicrobial effect on *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus subtilis,* and *Escherichia coli*. The results show that Hibiscus Wine MTCC 180, at concentrations (2,4 and 8 mg/ml) in table-2 had less significant antimicrobial effect on *Salmonella typhimurium Staphylococcus aureus*, *Bacillus subtilis,* and *Escherichia coli.* and it is not able to prevent the growth of bacteria on culture ($p<0.05$). However, (16, 32, and 64 mg/ml) Hibiscus Wine MTCC 180 concentrations, have significant antimicrobial effect on *Salmonella typhimurium Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*. The results show that Hibiscus Wine MTCC 786, at concentrations (2,4 and 8 mg/ml) in table-3 had less significant antimicrobial effect on *Salmonella typhimurium Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli.* and it is not able to prevent the growth of bacteria on culture ($p<0.05$). However, (16, 32 and 64 mg/ml) Hibiscus Wine MTCC 786 concentrations, have significant antimicrobial effect on *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus subtilis,* and *Escherichia coli*.

All the samples showed zones of inhibition around 11-16 mm against food borne pathogens which has proved the antimicrobial activity of Hibiscus wine.

Table-1: Minimum Inhibitory Concentration (MIC) of Hibiscus wine prepared from *Saccharomyces cerevisiae* MTCC 178 on *Bacillus subtilis, Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus*

	Hibiscus wine concentrations (mg/ml)					
Microorganisms	**2**	**4**	**8**	**16**	**32**	**64**
Bacillus subtilis	-	-	-	+	+	+
Escherichia coli	-	-	-	+	+	+
Salmonella. typhimurium	-	-	-	+	+	+
Staphylococus aureus	-	-	-	+	+	+

Table-2: Minimum Inhibitory Concentration (MIC) of Hibiscus wine prepared from *Saccharomyces cerevisiae* MTCC 180 on *Bacillus subtilis, Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus*

	Hibiscus wine concentrations (mg/ml)					
Microorganisms	**2**	**4**	**8**	**16**	**32**	**64**
Bacillus subtilis	-	-	-	+	+	+
Escherichia coli	-	-	-	+	+	+
Salmonella typhimurium	-	-	-	+	+	+
Staphylococcus aureus	-	-	-	+	+	+

Table-3: Minimum Inhibitory Concentration (MIC) of Hibiscus wine prepared from *Saccharomyces cerevisiae* MTCC 786 on *Bacillus subtilis, Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus*

	Hibiscus wine concentrations (mg/ml)					
Microorganisms	**2**	**4**	**8**	**16**	**32**	**64**
Bacillus subtilis	-	-	-	+	+	+
Escherichia coli	-	-	-	+	+	+
Salmonella typhimurium	-	-	-	+	+	+
Staphylococcus aureus	-	-	-	+	+	+

+ Positive inhibition.
- Negative inhibition.

Table-4: Average diameter (mm) of microbial free zone area of Hibiscus wine (at different concentrations) prepared from *Saccharomyces cerevisiae* MTCC 178 on *Bacillus subtilis, Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus*

Samples	**Zone of inhibition(mm)**			
Hibiscus wine MTCC 178	***Bacillus subtilis***	***Escherichia coli***	***Salmonella typhimurium***	***Staphylococcus aureus***
2	2.10	2.00	2.11	2.29
4	2.80	2.10	2.14	2.50
8	2.44	2.90	2.10	2.00
16	13.10	13.00	13.10	13.10

32	15.00	14.90	14.15	15.11
64	15.00	15.90	15.00	15.20

Table-5: Average diameter (mm) of microbial free zone area of Hibiscus wine (at different concentrations) prepared from *Saccharomyces cerevisiae* MTCC 180 on *Bacillus subtilis, Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus*

Samples	Zone of inhibition(mm)			
Hibiscus wine MTCC 180	***Bacillus subtilis***	***Escherichia coli***	***Salmonella typhimurium***	***Staphylococcus aureus***
2	1.22	1.10	1.55	1.39
4	1.82	1.30	1.27	1.79
8	1.44	1.40	1.19	1.00
16	12.10	11.22	11.69	12.90
32	13.12	13.20	13.11	12.20
64	15.10	15.10	14.22	14.25

Table-6: Average diameter (mm) of microbial free zone area of Hibiscus wine (at different concentrations) prepared from *Saccharomyces cerevisiae* MTCC 786 on *Bacillus subtilis, Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus*

Samples	Zone of inhibition(mm)			
Hibiscus wine MTCC 786	***Bacillus subtilis***	***Escherichia coli***	***Salmonella typhimurium***	***Staphylococcus aureus***
2	1.20	1.00	1.10	1.50
4	1.90	1.10	1.30	1.90
8	1.40	1.00	1.35	1.50
16	12.00	11.12	11.38	11.90
32	11.00	11.30	11.80	11.50
64	12.10	12.30	12.10	12.20

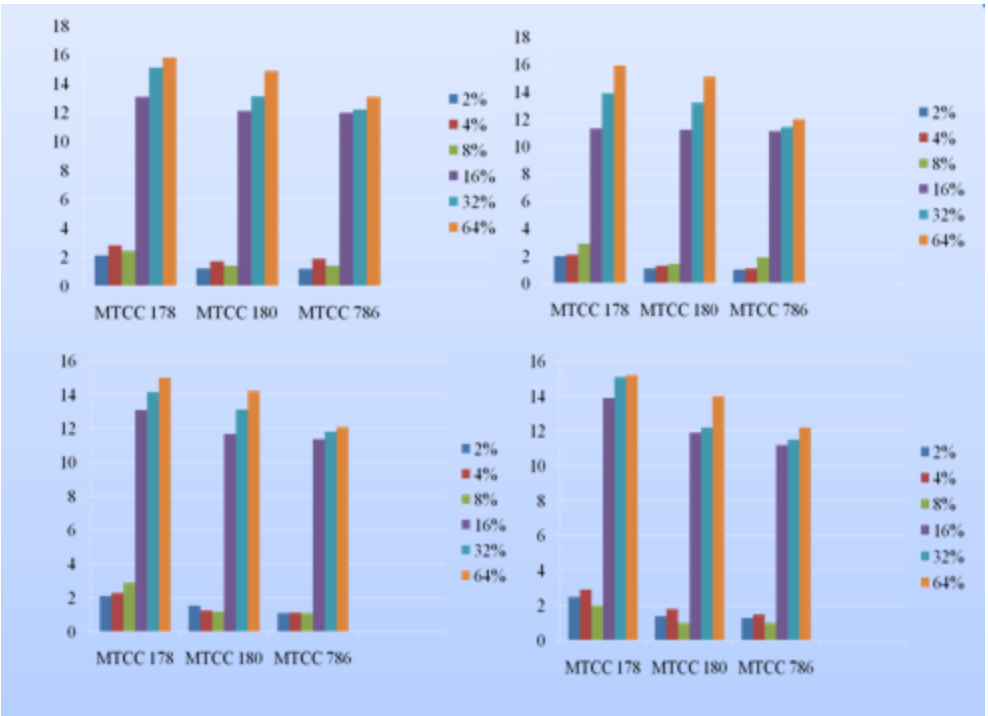

Fig-1 MIC of *Bacillus subtilis, E.coli , Salmonella typhimurium & Staphylococcus aureus,* for different yeast strains of *Saccharomyces cerevisiae* in *Hibiscus rosa-sinensis* wine respectively

The antimicrobial activities of the wine prepared from of *H. rosa-sinensis* flowers were carried out against food borne pathogens such as ***Bacillus subtilis, E.coli, Salmonella typhimurium & Staphylococcus aureus*** as shown in fig.1. with the help of fig. 1 we can see that Hibiscus wine prepared from MTCC 786 was not effective as much as MTCC 178 in respect of all pathogens.

Based on the results Hibiscus wine prepared from different strains in this study have significant antimicrobial activity on the studied microorganisms. Tables (1,2,3,4,5,6) given above are representative of the MIC of *H. rosa-sinensis wine*. The growth of all pathogens were inhibited at different concentrations of wine, while the bactericidal effect of the herb varies for all organisms. all wines of different yeast strains showed promising zone of inhibition of growth in the range of 11.00±0.17 to 16.00 ±0.10 mm.

CONCLUSION

Our results are of great practical importance as the prepared herbal wines besides being a tasteful addition to food might also prove to be a health drink with antimicrobial potential against a variety of food-borne pathogens. samples showed zones of inhibition around 11-16

mm against food borne pathogens which has proved the antimicrobial activity of Hibiscus wine. The results of this present study have shown that Hibiscus petals has therapeutic potentials and contain bio-components. The activity of the Hibiscus flower wine may be indicative of the presence of broad spectrum bioactive compounds in the petals. Therefore, *Hibiscus rosa-sinensis* could be a promising natural antimicrobial agent with potential applications in pharmaceutical industry for controlling infections caused by the organisms used in this study. The prepared Hibiscus based herbal wines were found to possess bactericidal effect against common food-borne pathogens, Our results are of great practical importance as the prepared herbal wines besides being a tasteful addition to food might also prove to be a health drink with antibacterial potential against a variety of food-borne pathogens.

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