3.1. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Microorganisms

*Saccharomyces cerevisiae* MTCC 786 procured from Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India was used to carry out the fermentation. *Salmonella enteric* serovar Typhimurium (Virulent strain NCTC 74) (*S. Typhimurium*) was procured from the Central Research Institute (CRI), Kasauli (India), *Escherichia coli* NCIM 2065 was procured from National Collection of Industrial Microorganisms (NCIM), Pune (India). *Staphylococcus aureus* ATCC 9144, Standard probiotic strains of *Lactobacillus casei* MTCC 1423, *Lactobacillus plantarum* MTCC 2621 and *Lactobacillus acidophilus* MTCC 447, were procured from Microbial Type Culture Collection, IMTECH, Chandigarh, India.

3.1.2. Maintenance of microorganisms

Yeast strain of *S. cerevisiae* was maintained on PDA (Potato dextrose agar), (HiMedia, ME096, India), slants. The strain of *S. Typhimurium* was maintained on Mac Conkey agar, (HiMedia, M082, India), as well as nutrient agar (HiMedia, M002, India) slants while *S. aureus* and *E. coli* was maintained on nutrient agar slants. *Lactobacillus* strains were maintained on De Mann Rogosa Sharpe (MRS) agar (HiMedia, M369, India) slants. These slants were stored at 4°C and subcultures from the slants were made every month. For long term storage, glycerol stocks were prepared and kept at -70°C. Before use, the organisms were subcultured in respective broths, followed by plating and then isolated colony was picked up for further processing.

3.1.3. Animals and Ethics

Male BALB/c (4-6 weeks old) mice with a body weight ranging from 20 to 25 gm, were procured from the Central Animal House of Panjab University, Chandigarh, India. These were housed in polypropylene cages (10 mice per cage) bedded with clean rice husk in
well-aerated animal room of the Department of Microbiology. All the animals were fed with the standard pellet diet comprising of 20-21% crude protein, 4% fat, 5.0-5.75% crude fibre, 8-9% ash, 1.0-1.5% calcium, 0.6-0.8% phosphorus and 50% nitrogen free extract (M/s. Ashirwad Industries Pvt. Ltd., Punjab, India) and water ad libitum. Animals were acclimatized to the new housing and experimental conditions for at-least one week. The experimental protocols were approved by the Institutional Animal Ethics Committee of Panjab University, Chandigarh, India (Registration number: 45/1999/CPCSEA) and performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, on animal experimentation. All efforts were made to minimize suffering of the animals.

3.1.4. Glassware and plasticware

Throughout the study glassware from Borosil and Schott Duran, plasticware from Tarsons and Laxbro were used after washing with the detergent and finally rinsed in distilled water. These were then dried in hot air oven at 70°C before use.

3.1.5. Chemicals and reagents

All the chemicals and reagents used in the present study were of high quality and purity grade obtained from E-Merck, Hi-Media, SD Fine Chemicals, Qualigens, and SRL (India). ERBA test kits (ERBA Diagnostics, Mannheim, Germany), were used for measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities.

3.1.6. Raw substrates for wine production

_Aloe vera_ (Aloe barbadensis Miller) leaves were collected from local nursery, amla fruits, ginger fresh red grapes, were collected from the local market of Chandigarh city. These were washed in clean water and dried in air.
3.2. METHODS

3.2.1. Process development for the fermentation of *Aloe vera* gel

The process for fermenting *Aloe vera* gel involved the following steps:

3.2.1.1. Extraction and processing of *Aloe vera* gel

*Aloe vera* leaves were collected from local nursery in Chandigarh and the colorless gel contained in the inner part of the fresh leaves extracted by hand filleting method (Ramachandra and Srinivasa Rao, 2008) was used for the production of wine. In order to avoid the loss of biological activity filleting operation was completed within 24 h of harvesting the leaves. Briefly, the lower 1 inch of the leaf base, the tapering point (2-4 inches) of the leaf top and the short sharp spines located along the leaf margins were removed by a sharp knife. The knife was inserted into the mucilage layer below the green rind followed by the removal of top and bottom rinds. The gel was then blended in a mixer and the resulting *Aloe vera* juice is stored in amber colored glass bottles to avoid the effect of light on the sensitive bioactive agents. The total soluble solids (TSS) of *Aloe vera* gel was adjusted to 20°B with cane sugar and the pH was adjusted to 4.5 using citric acid.

3.2.1.2. Inoculum preparation

25 mL of sterilized glucose yeast extract (GYE) broth (yeast extract 0.3% w/v, malt extract 0.3% w/v, peptone 0.5% w/v and glucose 1% w/v, pH 4.5), dispensed in 100 mL flask was inoculated with loopful culture of *S. cerevisiae* from a slant. The flask was incubated at 30°C on a rotary shaker (150 rpm) for overnight and the cells were separated by centrifugation at 10000 rpm (4°C, 15min). These were washed twice and re-suspended in normal saline to give a concentration of 1×10^8 cells/mL which was used as a pre-inoculum. The inoculum was prepared by transferring 10 mL of the pre-inoculum to 250 mL conical flask having 100 mL of *Aloe vera* juice supplemented with 5% sucrose and incubating overnight as shake culture (150 rpm) at 30°C.

3.2.1.3. Fermentation of *Aloe vera* gel

*Aloe vera* gel supplemented with cane sugar was subjected to batch fermentation. One litre of gel medium having 20°B TSS with 19% (w/v) total sugar was taken in 2 L Erlenmeyer flask, seeded with 10% (v/v) inoculum having a viable count of 1×10^8 cells/mL,
supplemented with 100 ppm sodium metabisulphite and incubated in a stationary state after plugging with cotton wool in a BOD incubator at 30°C for 10 days. The contents of the flask was mixed 2-3 times a day and the progress in fermentation was noted at regular intervals of 24h by analyzing total soluble solids (TSS), pH, total sugar, ethanol as discussed below:

3.2.1.3.1. Estimation of Total soluble solids (°Brix)

Brix (°B) reading of the wine samples was determined using ERMA hand refractometer having a range of 0-32°Brix.

3.2.1.3.2. Estimation of pH

pH of the samples were recorded by using the pH meter (ME-962-P), Max Electronics, India. Standard buffer solutions of pH 4.0, 7.0 and 9.0 were used as reference to calibrate.

3.2.1.3.3. Estimation of Total sugars

The sugar content was detected by Fehling’s method (Fehling, 1849)

Reagents

Sodium potassium tartarate (NaKC₄H₆O₆) = 86.5 gm
Sodium hydroxide (NaOH) = 25 gm
Both above chemicals were mixed and distilled water was added to make a volume of 250 mL = Fehling A
Copper sulphate (CuSO₄) = 17.31 gm
In above chemical, distilled water was added to form a volume of 250 mL = Fehling B
Both Fehling A and B were taken and mixed with each other in equal volume for the estimation of total sugars.

Principle

The principal sugars utilized by yeast in alcoholic fermentation are glucose and fructose, referred to as reducing sugars as they are capable of reducing copper (as Cu(II)).
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Fehling's method is based on the principle that the reducing sugar will convert the blue alkaline cupric sulphate solution in boiling alkaline medium to a red precipitate of cuprous oxide.

Procedure:

For acid hydrolysis to convert disaccharides to their component reducing sugars, 5mL of sample was taken to which few drops of cone. HCl were added. It was kept at 70°C for 15-20 min in water bath for the hydrolysis of sugars present in sample. To make it alkaline 10% NaOH was added after adding few drops of 1% phenolphthalein till the appearance of pink colour. Then the final volume was make up to 50 mL. 5 mL Fehling A and 5 mL of Fehling B was taken in a flask. The content of the flask was boiled and the hydrolyzed sample was added drop wise until the blue colour has disappeared. Titration was carried on till brick red colour was obtained. 1% glucose was used as a standard and titration was done as explained above to know the amount of glucose required to completely reduce 10 mL of Fehling solution.

3.2.1.3.4. Estimation of ethanol

The ethanol was estimated by colorimetric method as described by Caputi et al., (1968).

Preparation of Potassium dichromate reagent

Potassium dichromate (K₂Cr₂O₇) 34.0 gm

Sulphuric acid (H₂SO₄) 325.0 mL

Dissolved potassium dichromate in 400 mL distilled water followed by addition of sulphuric acid to chilled potassium dichromate solution and the volume was made up to 1000 mL with distilled water.

Procedure

1 mL of representative samples of wine was transferred to 250 mL round bottom distillation flask connected to the condenser and was diluted with 30 mL distilled water. The
sample was distilled at 80-90°C. The distillate was collected in 25 mL of K₂Cr₂O₇ reagent, which was kept at receiving end. The distillate containing alcohol was collected till total volume of 45 mL was obtained. Similarly standards (1-10% ethanol) were mixed with 25 mL of K₂Cr₂O₇ separately. The distillate of samples and standards were heated in water bath at 70°C for 20 min and cooled. The volume was made up to 50 mL with distilled water and the optical density was measured at 600 nm using UV 1900 spectrophotometer. The standard curve was plotted considering the concentration against absorbance. (Appendix 1)

Fermentation efficiency was calculated as:

\[
\text{Fermentation efficiency (FE)} = \frac{\text{Alcohol content in wine}}{\text{Alcohol content obtainable from sugar utilized}} \times 100
\]

Alcohol obtainable from 1gm glucose, according to Gay-Lussac’s equation, is 0.511gm which comes out to be 0.64mL after considering the density of alcohol as 0.8.

3.3 Standardization of various process parameters for the production of Aloe vera wine

* Aloe vera* wine fermentation was optimized by standardizing various environmental and cultural conditions known to affect yeast growth and the alcohol production by varying one factor at a time. 100 mL *Aloe vera* juice was taken in different sets of 250 mL Erlenmeyer flasks. Cane sugar was added to adjust the TSS at 20°B and maintaining the pH at 5 with citric acid. These flasks were inoculated with a 10% (v/v) inoculum made with an overnight grown culture of *S. cerevisiae* as discussed in section 3.2.1.2 having a viable cell count of 1×10⁸/mL and were subjected to batch fermentation for 7 days and incubated at 30°C, unless otherwise stated.

3.3.1 Standardization of environmental factors

This was done by studying the effect of incubation temperature, pH of the medium, inoculum size and sugar concentration in the medium.

- The effect of temperature was studied by incubating the *Aloe vera* based production media, after inoculation, at different temperatures including 15°C, 20°C, 25°C, 30°C, 35°C and 40°C.
The effect of pH was studied by varying the pH (3.5-6.0) of the production media using citric acid or sodium bicarbonate.

The effect of inoculum level was studied by inoculating the production media with varying levels of inoculums (2-12% v/v) having $10^8$ viable cells/mL.

The effect of sugar concentration was studied by adjusting the solid content in the production media at 10, 15, 20 and 25°B with cane sugar.

### 3.3.2. Standardization of nutritional factors

This was attempted by incorporating various nitrogen sources and metal salts in the production medium.

- The effect of nitrogen sources was studied by incorporating ammonium sulphate ($\text{NH}_4\text{SO}_4$), urea, diammonium hydrogen phosphate ($\text{(NH}_4\text{)}_2\text{HPO}_4$), malt extract, yeast extract, soyabean meal, corn steep liquor or peptone, separately at a concentration of 0.1% (w/v) in the *Aloe vera* based production medium.

- The effect of metal salts was studied by supplementing magnesium sulphate ($\text{MgSO}_4\cdot7\text{H}_2\text{O}$), calcium chloride ($\text{CaCl}_2\cdot2\text{H}_2\text{O}$), sodium chloride ($\text{NaCl}$), potassium chloride ($\text{KCl}$), zinc sulphate ($\text{ZnSO}_4\cdot7\text{H}_2\text{O}$) or potassium dihydrogen orthophosphate ($\text{KH}_2\text{PO}_4$), separately, at a level of 0.1% (w/v) in the *Aloe vera* based production medium.

### 3.4. Production of *Aloe vera* wine under optimized process conditions

*Aloe vera* wine was produced by selecting the optimized fermentation parameters and following the steps mentioned in section 3.2.1. In brief, *Aloe vera* gel supplemented with cane sugar, ($\text{(NH}_4\text{)}_2\text{HPO}_4$, $\text{MgSO}_4\cdot7\text{H}_2\text{O}$ and $\text{KH}_2\text{PO}_4$ (0.1% w/v) each with pH 4.5 was subjected to batch fermentation. One litre of medium containing 20°B TSS with 19% (w/v) total sugar was taken in 2L Erlenmeyer flask, seeded with 10% (v/v) inoculum, supplemented with 100 ppm sodium metabisulphite and incubated in a stationary state after plugging with cotton wool in a BOD incubator at 30°C till no further decrease in °Brix level was noted. The contents of the flask was mixed 2-3 times a day and the progress in fermentation was noted at regular intervals of 24h by analyzing TSS, pH, sugar content and
ethanol as described in section 3.2.1.3. After completion of fermentation, the wine was clarified, by repeated siphoning which was carried out 4 times with a sedimentation period of 3 days between each siphoning. The wine was analyzed quantitatively for various constituents including total acids, soluble proteins, higher alcohols, esters, total phenolics, antioxidant activity and elements including Ca, Na, K, Mg, Fe, Cu, Zn, Mn. Phytochemical screening of Aloe vera wine was also done qualitatively including tannins, terpenoids, flavonoids, saponins, glycosides, alkaloids, polysaccharides and amino acids.

3.4.1 Analytical methods

3.4.1.1. Estimation of Titrable acidity

It was expressed as percent acidity and analyzed using method of Amerine et al., (1980). Titrable acidity was determined by titrating known amount of wine sample (10 mL) against 0.2 N NaOH using a few drops of 1% phenolphthalein solution as indicator. The end point was appearance of pink/purple colour which should persist for 15-20 sec.

\[
\text{Titrable acidity} = \frac{\text{Volume of NaOH used} \times 0.75}{10 \text{ mL (volume of sample taken)}}
\]

3.4.1.2. Estimation of Total soluble proteins

Protein content of the samples was estimated by the method of Lowry et al., (1951).

Principle

The protein in the sample when treated with alkaline copper tartarate solution forms a cupric amino acid complex. An intense blue colour is formed due to the reduction of phosphomolybdic acid and phosphotungstic acid by aromatic amino acid complexes. The absorbance of this blue coloured complex is measured at 680 nm.

Reagents

<table>
<thead>
<tr>
<th>Solution A</th>
<th>Solution B</th>
<th>Solution C</th>
</tr>
</thead>
<tbody>
<tr>
<td>: 2% Na₂CO₃ in 0.1 N NaOH.</td>
<td>: CuSO₄ (1%).</td>
<td>: NaKC₄H₄O₆ (2%).</td>
</tr>
</tbody>
</table>
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**Working solution**: 48 mL of solution A + 1 mL of solution B + 1 mL of solution C

**Folin Ciocalteu’s reagent**: Diluted with water (1:1, v/v).

**Standard**: Standard concentration in the range of 0-200μg with stock solution of BSA (20 mg/100mL) in distilled water.

**Procedure**

To 3.0 mL of working solution, 1.0 mL of the appropriately diluted sample (1:50 dilution) was added, vortexed and incubated at 37°C in water bath for 10 min. After incubation, 0.3 mL of diluted (1:1) Folin’s reagent was added and the mixture was vortexed again. The tubes were then incubated at 37°C in water bath for 30-45 min. The absorbance was read at 680 nm. Distilled water (1.0 mL) processed as above was used to blank the spectrophotometer. The standard curve was plotted considering the concentration against absorbance (Appendix 2).

**3.4.1.3. Estimation of other alcohols and esters**

Methanol, n-propanol, n-butanol, iso-amyl alcohol and ethyl acetate were detected and quantified by gas chromatography (GC) (Hewlett Packard 5790) equipped with flame ionizing detector using a glass column (6’×1/4”) packed with carbowax-20 M. The analyzing conditions were Gases: Nitrogen 25 mL/min, oxygen 94 mL/min and hydrogen 30 mL/min; temperature T1 40°C for 1 min, T2 200°C, injection temperature 250°C; Detector temperature 200°C; Injection volume: 1 μl, 2 μl and 3 μl. Wine samples were sent to M/S Jagatjit Industries Limited, Hamira, Punjab (India), to quantify the levels of other alcohols and esters in the prepared Aloe vera wines.

**3.4.1.4 Estimation of minerals**

The estimation of the concentration of minerals (Ca, Mg, Fe, Cu, Zn, Mn) was done by atomic absorption spectrophotometer (Perkin Elmer-3100) and (Na, K,) was estimated by flame photometer. Wine samples were sent to Agri and Food Testing Laboratory, Punjab Biotechnology Incubator, Mohali, Punjab, India to quantify the concentration of minerals.
3.4.1.5. Determination of total phenolic compounds

The total phenolics content of the wine was determined by the Folin–Ciocalteau colorimetric method (Waterhouse, 2002). The Folin-Ciocalteau method relies on oxidation-reduction reactions, and measures the total phenolic content as this colour forming reaction is produced by monohydric phenols, polyphenols, flavonoids, tannins, and some other readily oxidized substances such as ascorbic acid. The reagent, phosphotungstic-phosphomolybdic acid oxidises phenols and is itself reduced to a blue molybdenum tungsten complex that is then measured at 765 nm. Phenols are more rapidly oxidised in solutions sufficiently alkaline to give appreciable concentrations of the phenolate ion. The final phenolic concentration is determined by obtaining a standard curve with gallic acid (0.1mg/mL) as reference (Appendix 3).

Procedure:

To 1.0 mL of appropriately diluted wine sample, 1.0 mL of Folin - Ciocalteau reagent was added and the content was mixed thoroughly. After 3 min, 3 mL of 2% Na$_2$CO$_3$ was added then the mixture was allowed to stand for 2h with intermittent shaking. The absorbance was measured at 765 nm using spectrophotometer and expressed as mg gallic acid equivalents per litre of sample (mg GAE/L).

3.4.1.6. Antioxidant activity of wine

Total antioxidant activity of the wine was determined using the ferric reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996). In this assay, antioxidants are evaluated as reductants of Fe$^{3+}$ to Fe$^{2+}$, which is chelated by 2,4,6-tripyridyl-S-triazine (TPTZ) to form a Fe$^{2+}$-TPTZ complex absorbing at 740 nm. Measurements were done in triplicate. Results were compared with a standard curve prepared with different concentrations of FeSO$_4$.7H$_2$O (0.01-1µmol). The antioxidant efficiency of the sample solution was calculated with reference to the standard curve given by a Fe$^{2+}$ solution of known concentration. Ferric reducing power of the sample was expressed in µmol Fe$^{2+}$/L. The standard curve was plotted considering the concentration against absorbance (Appendix 4).
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**Reagents**

- **Acetate buffer**: 1.0 mol/L, pH 3.6
- **TPTZ reagent**: 10 mmol/L 2,4,6-tripyridyl-S-triazine in 40 mmol/L hydrochloric acid
- **Ferric chloride**: 20 mmol/L
- **Standard**: A standard curve was prepared of FeSO₄·7H₂O (0.01-1 μmol).

**Procedure**

The working FRAP reagent was prepared by mixing 10 volumes of 1.0 mol/L acetate buffer, pH 3.6 with 1 volume of 10 mmol/L TPTZ (2,4,6-tripyridyl-S-triazine) in 40 mmol/L hydrochloric acid and with 1 volume of 20 mmol/L ferric chloride. In a reaction tube, 100 μL of wine sample was added into 3.0 mL of FRAP reagent. Absorbance was measured at 740 nm after 10 min.

**3.4.1.7. Qualitative analysis of phytochemicals in Aloe vera wine**

The qualitative screening for common phytochemicals including tannins, terpenoids, flavonoids, saponins, glycosides, alkaloids, polysaccharides and amino acids was carried out in all the Aloe vera wines by standard methods as described below:

- **a) Tannins**: To 0.5 mL of test solution, 1 mL of water and 1-2 drops of 0.1% ferric chloride solution was added. Occurrence of a blue-black, green or blue-green precipitate indicates the presence of tannins (Trease and Evans, 2002).

- **b) Terpenoids**: 5 mL of test sample was mixed with 2 mL of chloroform. 3 mL of concentrated sulphuric acid was carefully added to the mixture to form a layer. An interface with a reddish brown coloration was observed for terpenoids (Krishnaiah et al., 2009).

- **c) Flavonoids**: 1 mL of test sample was treated with a few drops of 1% NH₃ solution. Yellow colour formation was an indication for the presence of flavonoids (Krishnaiah et al., 2009).
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**d) Saponins:** 10 mL of test sample was mixed with 5 mL of distilled water in a test tube and shaken vigorously to obtain a stable persistent froth. The frothing was then mixed with 3 drops of olive oil and observed for the formation of emulsion, indicating the presence of saponins (Krishnaiah et al., 2009).

**e) Glycosides:** To 1 mL of test solutions, 3 mL of anthrone reagent was added and mixed well. Formation of green coloured complex was a positive test (Sofowara, 1993).

**f) Alkaloids:** To the extract, few drops of Mayer’s reagent were added. White yellowish precipitate was observed for the presence of alkaloids. (Sofowara, 1993).

Mayer’s reagent: HgCl₂ - 1.36 gm
KI - 5 gm
Distilled water - 100 mL

**g) Polysaccharides:** To 1 mL of test solutions, 2 drops of iodine solution was added. Blue coloured solution observed confirmed positive test for polysaccharides (Sofowora, 1993).

**h) Free amino acids:** To 1 mL of test solutions, 5 drops of ninhydrin was added and boiled for 2 min. Formation of purple coloured solution indicates the presence of free amino acids (Sofowara, 1993).

3.4.2. Maturation of *Aloe vera* wine

Ageing of *Aloe vera* wine was done for one year in an oak wood barrel (2.5 L), procured from M/S Jagatjit Industries Limited, Hamira, Punjab (India). The container was filled up to the brim and analyzed for various components after one year of ageing by methods discussed in section 3.2.1.3 and 3.4.1.

3.5. Assessment of safety of prepared *Aloe vera* wine

The safety of prepared *Aloe vera* wine was assessed by its oral feeding in a mice group comprising of at least 10 mice and monitoring their renal and liver function tests as
well as liver histology. Each mouse in this group was orally fed with the standardized dose of 0.3 mL of *Aloe vera* wine once in a day for 4 weeks.

### 3.5.1. Measurement of indicators of kidney and liver function

Blood was collected from the retro-orbital plexus of wine fed mice using fine heparinized glass capillary. For separation of serum, blood samples were allowed to clot at room temperature (25-28°C) for 1-2 h. The clotted blood samples were centrifuged at 2500 rpm for 10 min to harvest clear supernatant as serum. Serum samples were sent to a clinical laboratory in PGIMER, Chandigarh, India to measure essential biochemical indicators of kidney and liver function including urea, creatinine, AST (aspartate aminotransferase), ALT (alanine aminotransferase), TB (total bilirubin), DB (direct bilirubin), ALP (alkaline phosphatase), albumin, globulin.

### 3.5.2. Liver histology

Livers were removed aseptically from mice administered with 0.3mL of wine once in a day for 4 weeks and rinsed in isotonic saline solution. A part of liver was then fixed in 10% buffered formalin and processed for histological examination as follows:

After fixation in formaldehyde, tissues were exposed to 70%, 90% and 100% ethanol for 1 h each. The last wash with 100% ethanol was repeated for 1h. The tissues were then transferred to xylene, 3 times, for 1 h each and embedded in molten paraffin wax maintained at 60°C. Liver tissue blocks were sectioned at 4 μm thickness using a fine blade attached to Spencer Type AO Rotary Microtome and kept in a section flotation water bath at 45°C. Sections were placed onto aluminized glass microscope slides coated with a mixture of equal volume of glycerine and Mayer’s egg albumin. Wax was removed completely by placing the slides in xylene for 5 min followed by successive treatments with fresh 100% ethanol for 1 min each. Slides were then rinsed with water and stained with hematoxylin for 3 min. The excess stain was washed with water and the slides were then counter-stained with 1% eosin solution for 3 min followed by washing with water for 1 min and blot-dried. These were mounted with a drop of Distyrene Plasticizer Xylene (DPX), allowed to dry and examined under 100x objective using the light microscope (Olympus, India Pvt. Ltd.; Model: CH20BIMF 200) for histological analysis. The slides were photographed by a camera (Magnus) fitted on the microscope and using a software Grand VCD 2000 Plus (version 3.2).
3.6. **Organoleptic evaluation of prepared *Aloe vera* wine**

The acceptability of *Aloe vera* wine was ascertained by a panel of 5 judges. Twenty point scale (Amerine and Ough, 1980) based on the appearance, colour, aroma, bouquet, acescent, total acid, sugar, body, flavor, astringency and general quality was used for sensory evaluation.

**Sample presentation for judges evaluation**

Prior to the presentation of the product, each sample was coded and placed in random manner, the different wine samples were placed along with a glass of water (to rinse the mouth) in the laboratory. The panelists were instructed to evaluate each sample by blind tasting as per score card.

**Sensory evaluation:**

The wine samples were evaluated by a panel of 5 judges. The numerical scoring method and the score card followed are given below:

**Score card**

<table>
<thead>
<tr>
<th>Code of the sample</th>
<th>Appearance</th>
<th>Colour</th>
<th>Aroma</th>
<th>Bouquet</th>
<th>Acescent</th>
<th>Total acidity</th>
<th>Sugar</th>
<th>Body</th>
<th>Flavour</th>
<th>Astringency</th>
<th>General quality</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>20</td>
</tr>
</tbody>
</table>
Grading according to score:

17-20, wines with outstanding characteristics and no marked defect; 13-16, standard wines with neither an outstanding character nor defect; 9-12, wines of commercial acceptability but with a noticeable defect; 5-8, wines of below commercial acceptability; 1-4, completely spoiled wines.

3.7. Production of variants of Aloe vera wine

Attempts were also made to add to the variety of Aloe vera wines by blending the Aloe vera based medium with some other herbs/fruits with bioactive components including amla, ginger and red grapes, separately. Aqueous decoction of amla and ginger was prepared by boiling 400 gm of intact amla fruits and sliced ginger pieces, separately, in 1000 mL of tap water for 15-20 min in a steel container. The container along with the contents was left undisturbed after covering it with a metal lid and allowed to cool. Grape juice was prepared by blending grapes in a mixer and the juice was stored in plastic bottles. All the substrates were mixed separately with 50% Aloe vera gel (v/v) and supplemented with cane sugar (TSS of 20°B), (NH₄)₂HPO₄, MgSO₄.7H₂O and KH₂PO₄ (0.1% w/v) each with pH 4.5. The resulting media were subjected to batch fermentation as discussed in section 3.2.1. till no further decrease in °Brix level was noted. The contents of the flask was mixed 2-3 times a day and the progress in fermentation was noted at regular intervals of 24 h by analyzing TSS, pH, sugar content and ethanol as described in section 3.2.1.3. After completion of fermentation, all the three wines were clarified, by repeated siphoning which was carried out 4 times with a sedimentation period of 3 days between each siphoning. As the matured Aloe vera wine was reported with better organoleptic characteristics relative to unmatured Aloe vera wine so the prepared variants of Aloe vera wine namely; Aloe-aml, Aloe-ginger, Aloe-grape were directly kept for maturation in oak wood barrels for a period of one year. The matured variants of Aloe vera wine were then analyzed for various components as discussed in section 3.2.1.3 and 3.4.1. The same were also evaluated for organoleptic characteristics as mentioned in section 3.6.
3.8. *In vitro* antibacterial efficacy of *Aloe vera* wines against common food-borne pathogens

The antibacterial efficacy of prepared *Aloe vera* wines against *S. Typhimurium*, *S. aureus* and *E. coli* was assessed by various techniques including assaying zone of inhibition by well diffusion, determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) values and time dependent bactericidal assay of different *Aloe vera* wines.

3.8.1. Zone of inhibition by well diffusion assay

Antibacterial activity of prepared wines against *S. Typhimurium*, *S. aureus* and *E. coli* was performed by well diffusion technique (Deans and Ritchie, 1987). The actively grown culture in nutrient broth was selected for plate assay with cell count of approximately $10^5$ cfu/mL. 100 µL liquid culture was spread on nutrient agar plate to create a bacterial lawn. Three wells with diameter of 6 mm were punched in each nutrient agar plate and 100 µL of either of *Aloe vera* wines was added to one of the wells in each plate under aseptic condition. 100 µL of unfermented *Aloe vera* based juice, as well as 10% (v/v) pure ethanol were also loaded, separately, in other two different wells in each plate as the controls to compare the antibacterial activity with prepared variants of *Aloe vera* wine namely, *Aloe vera* wine, *Aloe-amla* wine, *Aloe-ginger* wine and *Aloe-red grape* wine. The plates were left for 30 min at room temperature for the diffusion of the test samples before being incubated at 37°C for 24 h after which the diameter of zones of inhibition were measured. The analyses were carried out in triplicates.

3.8.2. Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of *Aloe vera* wines

MIC of wines for *S. Typhimurium*, *S. aureus*, *E. coli* was determined according to the method of Moretro and Daeschel, (2004). Different *Aloe vera* wines namely, *Aloe vera* wine, *Aloe-amla* wine, *Aloe-ginger* wine and *Aloe-red grape* wine were diluted separately, in brain heart infusion (BHI) broth (HiMedia, M210, India) to concentration in the range of 10% to 50% (5% interval, v/v), with a final volume of 3 mL in test tubes. BHI broth of double strength together with sterile distilled water was used to make dilutions, resulting in BHI with normal strength in final dilutions. The tubes were inoculated separately with 30 µL of
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either of the test organism culture grown overnight and incubated for 24 h at 37°C. MIC was determined as the lowest concentration of wine that inhibited the visible growth of bacteria after 24 h incubation. The MBC was determined by plating the contents of the tubes containing MIC and higher concentration of wine on nutrient agar plate and incubated at 37°C for 24 h. MBC was defined as the lowest concentration of wine resulting in >99.9% reduction of the initial inoculums. All analyses were carried out in triplicates.

3.8.3. Time dependent bactericidal assay

Bactericidal effect of prepared wines against S. Typhimurium, S. aureus, E. coli was studied as per the method of Moretro and Daeschel, (2004) separately for different wines. 10% (v/v) ethanol and Aloe based juices were used as controls. 50 µL of stationary phase (16 to 20 h) broth with approximately 10⁹ cfu/mL was used to inoculate 4.95 mL of either of the wines taken in 30 mL test tube (18×150 mm). The final cell concentration during exposure was approximately 10⁷ cfu/mL. The suspension was mixed with a vortex mixer and plated on to BHI agar after retention at ambient temperature for 0, 10, 20, 30 min. Plates were incubated for 24 h at 37°C and bacterial count was taken. All analyses were carried out in triplicates.

3.9.1 In-vitro effect of Aloe vera wines against probiotic bacteria

The effect of Aloe vera wines against probiotic strains namely, L. casei, L. plantarum and L. acidophilus was analyzed by agar well diffusion assay. 100 µL actively grown cultures in MRS broth of each of the strains with cell count of approximately 10⁸ cfu/mL was spread separately on MRS agar plate to create a bacterial lawn. Wells (6 mm dia) punched in each plate was filled aseptically with 100 µL of either of prepared wines. Thereafter, the plates were left for 30 min at room temperature before being incubated at 37°C for 24 h. The diameters of the zones of inhibition if any, were measured after 24 h. All analysis was carried out in triplicates.

3.9.2. Effect of oral administration of Aloe vera wines on resident microflora of mice

As Aloe vera wines were not found to be antibacterial against the useful probiotic strains of Lactobacilli, the safety potential of Aloe vera wines was also assessed in terms of persistence of natural gut flora by determining the microbial cell count in the fecal matter of
mice after oral administration of a standardized dose of 0.3 mL of either of the prepared *Aloe vera* wines once in a day continuously for 4 weeks. To enumerate the lactobacilli, freshly voided feces were collected and pooled from each mouse (0.5 gm/mouse) at 0, 7, 14, 21 days to confirm the non-inhibitory effect of *Aloe vera* wines on the gut microflora. The feces were homogenized in normal saline, serially diluted and plated on MRS agar for the enumeration of lactobacilli. The plates were incubated at 37°C for 24 h and the number of colonies appearing on the plates was recorded (Oyetayo *et al.*, 2003). All analyses were carried out in triplicates.

### 3.10 Assessment of therapeutic potential of *Aloe vera* wines in a murine model of *Salmonellosis*

Therapeutic efficacy of *Aloe vera* wines was assessed against *Salmonella* infection by different markers such as:

- Bacterial load in liver, spleen and intestine.
- Histological studies.
- Liver markers – ALT, AST.

To investigate the therapeutic effect of wine administration through oral route mice were divided into following four groups, each comprising of at least 10 mice for all the variants of *Aloe vera* wine.

1. **Control group**: In this group mice were administered normal saline orally for ten consecutive days.

2. **Wine per se group**: Each mouse in this group was orally fed with the standardized dose of 0.3 mL of either of wines separately, once in a day for ten consecutive days.

3. **Salmonella-Infected group**: Mice were challenged with a single oral dose of 0.2 mL of *S. Typhimurium* suspension with a viable count of $2.5 \times 10^7$ cfu/mL (Rishi *et al.*, 2007).
4. **Infected-Wine fed group:** Each mouse in the group was given a single challenge dose of 0.2 mL of *S. Typhimurium* (2.5 × 10^7 cfu/mL) and then each mouse was orally fed with the standardized dose of 0.3 mL of either of wines separately, once in a day for ten consecutive days.

### 3.10.1. Bacterial translocation in the liver, spleen and intestine

Liver, spleen and intestine of each mouse from group 3 and 4 were taken out aseptically, rinsed in 0.05 M phosphate buffered saline (PBS) (pH 7.4) and weighed. A 10% (w/v) tissue homogenate was prepared in 0.05 M PBS (pH 7.4) using a potter Elvehjem homogenizer. Serially diluted homogenates (0.1 mL) were plated separately on bismuth sulphite agar (BSA) (HiMedia, M027, India). Colonies were counted after incubation at 37°C for 24 h.

### 3.10.2. Histological analysis to determine the effect of administration of *Aloe vera* wines on liver damage caused due to Salmonellosis

Liver tissues removed aseptically from all the groups were cut into small pieces, fixed, stained with hematoxylin-eosin and examined as explained above in section 3.5.2.

### 3.10.3. Evaluation of liver function by assessing serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity

The serum obtained was used for the estimation of ALT and AST activities using ERBA test kits (ERBA Diagnostics, Mannheim, Germany).

#### 3.10.3.1 Determination of alanine aminotransferase (ALT) activity

**Principle**

The test is based on the rate of conversion of L-alanine and oxoglutarate into pyruvate and L-glutamate in the presence of enzyme alanine aminotransferase.

\[
\text{L-alanine} + 2-\text{Oxoglutarate} \rightarrow \text{Pyruvate} + \text{L-glutamate}
\]
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Procedure

To 1.0 mL of ALT reagent, 0.1 mL of serum sample was added, mixed and change in absorbance per min (ΔA/min) was read at 340 nm. Enzyme activity was expressed as International Units/ litre (IU/L).

Calculations

Absorbance change is converted into International Units (IU) of activity using the formula:

\[
\text{Activity of ALT (IU/L)} = \frac{(\Delta A/ \text{min.}) \times 10^3}{\frac{T.V.}{S.V.} \times \text{Absorptivity} \times P}
\]

Where:

- T.V. = Total reaction volume
- S.V. = Sample volume
- Absorptivity = Millimolar Absorptivity of NADH at 340 nm = 6.22
- P = Cuvette light path (cm) = 1 cm

Activity of ALT (IU/L) = \((\Delta A/ \text{min.}) \times \text{Factor (1768)}\)

3.10.3.2 Determination of aspartate aminotransferase (AST) activity

Principle

The test is based in the rate of conversion of L-aspartate and 2-Oxoglutarate into oxaloacetate and L-glutamate in the presence of enzyme aspartate aminotransferase.

\[
\text{L-aspartate} + 2-\text{Oxoglutarate} \rightarrow \text{Oxaloacetate} + \text{L-glutamate}
\]

Procedure

To 1.0 mL of AST reagent, 0.1 mL of serum sample was added, mixed and change in absorbance per min (ΔA/min) was read at 340 nm. Enzyme activity was expressed as IU/L.
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Calculations

Absorbance change is converted into International Units (IU) of activity using the formula:

\[
\text{Activity of AST (IU/L)} = \frac{(\Delta A/ \text{ min.}) \times T.V. \times 10^3}{S.V. \times \text{Absorptivity} \times P}
\]

Where:

- T.V. = Total reaction volume
- S.V. = Sample volume
- Absorptivity = Millimolar Absorptivity of NADH at 340 nm = 6.22
- P = Cuvette light path (cm) = 1 cm

Activity of AST (IU/L) = \((\Delta A/ \text{ min.}) \times \text{Factor (1768)}\)

3.11. Antioxidative potential of the prepared Aloe vera wines

All the Aloe vera wines were evaluated for their antioxidative potential by determining the following parameters in the liver homogenates of the murine model:

- Extent of lipid peroxidation (Wills, 1966).
- Activity of superoxide dismutase (SOD) (Kono, 1978).
- Levels of reduced glutathione (GSH) (Jollow et al., 1974).

3.11.1. Preparation of liver homogenate and post mitochondrial supernatant (PMS)

Livers removed aseptically from all the above mentioned animal groups were rinsed in 0.05 M PBS, pH 7.4 and weighed. For every animal, a 10% (w/v) tissue homogenate was prepared in PBS (0.05 M, pH 7.4) using a Potter-Elvehjem homogenizer. An aliquot of the liver homogenate was used for the estimation of lipid peroxidation. For the estimation of superoxide dismutase and reduced glutathione levels, post mitochondrial preparation was prepared. For this, the liver homogenates were centrifuged at 10000 rpm for 20 min at 4°C. The supernatants thus obtained were called post mitochondrial supernatants (PMS).
3.11.2 Extent of peroxidative damage in liver

Quantitative measurement of lipid peroxidation in liver was performed according to the method of Wills, (1966).

**Principle**

Lipid peroxidation is a marker to assess oxidative damage caused by reactive oxygen species. Malondialdehyde (MDA), formed from the breakdown of polyunsaturated fatty acids, serves as an index for determining the extent of the peroxidation reaction. MDA has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid (TBA) to give pink colored product whose absorbance is measured at 532 nm.

**Reagents**

- Tris-HCl buffer : 0.1M Tris-HCl, pH 7.4
- Trichloroacetic acid (TCA) : 10% (w/v)
- Thiobarbituric acid (TBA) : 0.67% (w/v)

**Procedure**

To 0.5 mL of liver homogenate, 0.5 mL of Tris-HCL buffer (0.1 M, pH 7.4) was added and the mixture was incubated at 37°C for 2 h. Following incubation, 1.0 mL of 10% (w/v) ice-cold trichloroacetic acid was added and the mixture was centrifuged at 800 x g for 10 min. To 1.0 mL of the supernatant (obtained after centrifugation), 1.0 mL of 0.67% (w/v) TBA was added and the mixture was kept in boiling water bath for 10 min. After cooling the tubes, 1.0 mL of distilled water was added and absorbance was measured at 532 nm on a spectrophotometer (Hitachi U-1900, Japan). The results were expressed as nanomoles of MDA per milligram of protein (as calculated by the method of Lowry et al., (1951), described in section 3.4.1.2., using the molar extinction coefficient of the chromophore, MDA (1.56 x 10^5 M^-1 cm^-1)).
3.11.3. Determination of superoxide dismutase (SOD) levels

SOD activity was assayed according to the method of Kono, (1978).

Principle

The method is based on the principle of inhibitory effects of the SOD on the reduction of nitrobluetetrazolium (NBT) dye by superoxide anions generated by the auto-oxidation of hydroxyl amine hydrochloride. The addition of NBT to the reaction mixture stimulates oxidation of hydroxylamine and subsequent reduction of NBT leading to an increase in the absorbance at 560 nm. Dismutase activity of the SOD enzyme traps the superoxide radicals, hence, inhibiting the reduction of NBT to the blue colored compound, formazan.

Reagents

- Solution A: 50 mM Na$_2$CO$_3$ containing 0.1 mM EDTA; pH 10.8
- Solution B: 96 µM NBT in solution A
- Solution C: 0.6% Triton X-100 in solution A
- Solution D: 20 mM hydroxylamine HCl, pH adjusted to 6.0 with 0.1 N NaOH

Procedure

The reaction was initiated by the addition of 0.1 mL of hydroxyl amine hydrochloride (solution D) to the reaction mixture containing 1.3 mL solution A, 0.5 mL solution B, 0.1 mL solution C, and 10 µL PMS of liver homogenate. Change in absorbance at 560 nm was recorded for 3 min at 30 sec intervals.

3.11.4. Determination of reduced glutathione (GSH) levels

Reduced glutathione (GSH) levels in the liver were estimated according to the method given by Jollow et al., (1974).
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Principle

Reduced glutathione is a non-protein sulphydryl compound. DTNB (5, 5'-dithiobis-2-nitrobenzoic acid) is a disulfide compound that is readily reduced by sulphydryl compounds leading to the formation of a yellow-coloured compound, 2-nitro-5-mercaptobenzoic acid. The absorbance of this substance is measured at 412nm.

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>0.1 M phosphate buffer (pH 8.0)</td>
</tr>
<tr>
<td>Sulphosalicylic acid</td>
<td>4% (w/v) in distilled water</td>
</tr>
<tr>
<td>Ellman’s reagent</td>
<td>0.01 M 5, 5'-dithiobis- (2-nitrobenzoic acid)</td>
</tr>
</tbody>
</table>

Ellman’s reagent is 0.01 M 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) in 0.1 M phosphate buffer (pH 8.0).

Procedure

The liver homogenate (0.75 mL) was precipitated with 0.75 mL of 4% sulphosalicylic acid. The samples were kept at 4°C for at least 1h, and then subjected to centrifugation at 358 x g for 15 min at 4°C. The assay mixture contained 0.5mL of supernatant obtained above and 4.5 mL of Ellman’s reagent in a total volume of 5mL. The mixture was kept at room temperature for 10 min. The yellow colour developed was read immediately at 412 nm on a spectrophotometer. The results were expressed as micromoles of GSH per milligram of protein using the molar extinction coefficient of the chromophore, 2-nitro-5-mercaptobenzoic acid (13600 M⁻¹ cm⁻¹).

3.12. Effect of wine administration on Ferric reducing antioxidant power (FRAP) levels in plasma

The antioxidant capacity of plasma was characterized by FRAP assay, which measures the reduction of ferric iron to ferrous form in the presence of antioxidant components (Benzie and Strain, 1996). Blood was collected in eppendorfs containing heparin (30 IU/mL) from the retro-orbital plexus of all groups of mice using fine heparinized glass capillary. For separation of plasma, blood cells were removed by centrifugation at 6000 rpm at 4°C for 15 min. The clear supernatant was designated as plasma. 100 μL of plasma was used as a sample and the assay was carried out as described in section 3.4.1.6.
3.13. Morphological changes in the cells of *S. Typhimurium* in the presence of *Aloe vera* wine

The ultrastructural changes if any, induced by the treatment with *Aloe vera* wine were examined by scanning electron microscopy (SEM) according to the method of Singh *et al.*, (2013) with modifications. For the SEM study, single colony of *S. Typhimurium* was inoculated into 10 mL of BHI broth and the tube incubated at 37°C for 24 h. 1 mL overnight grown culture was then inoculated into a 10 mL tube of BHI which was used as control and incubated at 37°C for 24 h. Two 10 mL tubes of BHI containing 50% (v/v) *Aloe vera* wine was also inoculated with 1 mL overnight grown culture and incubated at 37°C for 6 h and 24 h respectively. Treated samples and control were centrifuged at (800 rpm for 20 mins); pellets were resuspended and fixed in 2% glutaraldehyde (1 hour at room temperature), dehydrated in a series of graded alcohol baths (50%, 10 min; 70%, 10 min; 80%, 15 min; 90%, 15 min; and 100%, 20 min), transferred on to glass cover slip and then subjected to air drying. Finally, the cover slips containing samples were mounted on aluminium stubs, coated with gold-palladium at a thickness of 200 Å and examined for the change in morphology by scanning electron microscope (Hitachi S-3400N model).

STATISTICAL ANALYSIS

The data were expressed as mean ± standard deviation (SD). The bacterial count was expressed as log_{10}. Statistical analysis was done by Student's unpaired *t* test and one-way analysis of variance (ANOVA), followed by pairwise comparison procedures (Tukey test), using Jandel Sigma Stat statistical software, version 2.0. In all cases, statistical significance was defined as having a *p* value of <0.05.