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Antibacterial activity of prepared *Aloe vera* based herbal wines against common food-borne pathogens and probiotic strains

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Abstract
Wine has been considered as safe and healthy drink, besides an important adjunct to the diet. The present work was carried out to develop process methodology for the production of *Aloe*-amla and *Aloe*-ginger wine. The prepared *Aloe vera* based herbal wines were evaluated for their effect against common food-borne pathogens and probiotic strains. *Aloe vera* gel, blended with amla and ginger extract and supplemented with sugar proved to be a good medium for the growth of *Saccharomyces cerevisiae* for making the *Aloe*-amla and *Aloe*-ginger wine. The wine was found to be similar to any other wine in terms of its composition and sensory quantities. The wine exhibited bactericidal activity against common food-borne pathogens (*S.* Typhimurium, *S. aureus, E. coli*). Captivatingly, the wine was not inhibitory to the selected probiotic strains and no significant difference in the viable count of lactobacilli was found in the fecal matter, hence, indicating their persistence in the gut of wine fed animal. It is suggested that the *Aloe vera* based herbal wines, as prepared in the present study, can be regularly consumed as a functional drink.

Keywords: *Aloe*-amla wine, *Aloe*-ginger wine, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, *Escherichia coli*, antibacterial activity.

1. Introduction
To improve the quality of life, extensive investments have occurred for the therapeutic applications of herbal plant-resources. Herbal preparations have been known to treat various infectious diseases throughout the history of mankind [1, 2]. Wine represents one of the functional fermented foods [3] and consumption of wine has been reported to exhibit protective effect, as regards the tendency to develop bacterial food infections [4]. It provides relaxation necessary for proper digestion and absorption of food and hence serves as a vital adjunct to the human diet [5]. Strong antibacterial activity of wine is its essential biological function which has been verified under various experimental conditions [6-12]. Wine serves as a base for medicinal preparations compounded with a range of herbs adapted to treat various disorders [13]. Functional botanical ingredients are more admired than ever in the beverage market. Many wines are made from herbs with perceived medicinal value and such wines have many additional health benefits. *Aloe vera* (*Aloe barbadensis* Miller), a well known herbal plant has been demonstrated to possess strong antibacterial, anti-inflammatory, anti-tumor and immune stimulatory properties. It has been utilized extensively in the preparation of health drinks [14]. The Indian gooseberry, also known as Amla (*Emblica officinalis*) have been reported to possess hypoglycemic, spasmolytic, purgative and antibacterial activity [15]. Several value added products have been prepared from Amla. Amla berries can be used as a valuable ingredient for the production of an herbal beverage [16, 17]. Ginger (*Zingiber officinale*) is well known for its contribution to food, and has antimicrobial [18] and antioxidant potentials [19, 20]. There are hundreds of beneficial compounds contained in these herbs which can deliver antimicrobial, antioxidant, anti-inflammatory, anti-mutagenic properties to the final food product [21]. This fact has encouraged us to explore the possible use of *Aloe vera*, amla and ginger for the production of functional wines and to evaluate the antibacterial potential of *Aloe vera* based herbal wines.

2. Materials and methods

2.1 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 enterica* 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.

2.2 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.

2.3 Raw substrates for wine production

Aloe vera (*Aloe barbadensis* Miller) leaves were collected from local nursery, amla fruits, ginger, were collected from the local market of Chandigarh city. These were washed in clean water and dried in air.

2.4 Extraction and processing of herbal substrates

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 [22] was used for the production of wine. Filleting operation was completed within 24 h of harvesting the leaves in order to avoid the loss of biological activity. Briefly, the lower leaf base, the tapering point of the leaf top and the short sharp spines placed 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 in order to avoid the adverse effect of light on the sensitive bioactive agents. 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. Both amla and ginger decoction was mixed separately with 50% *Aloe vera* gel (v/v) and supplemented with cane sugar (TSS of 20^{o}B), $(NH_4)_2HPO_4$, $MgSO_4.7H_2O$ and KH_2PO_4 (0.1% w/v) each with pH 4.5.

2.5 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 ^{o}C on a rotary shaker (150 rpm) for overnight and the cells were separated by centrifugation at 10000 rpm (4 ^{o}C, 15min). These were washed twice and re-suspended in normal saline to give a concentration of 10^8cells/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 ^{o}C.

2.6 Fermentation of *Aloe vera* based herbal media

Both *Aloe*-amla and *Aloe*-ginger media supplemented with cane sugar, $(NH_4)_2HPO_4$, $MgSO_4.7H_2O$ and KH_2PO_4 (0.1% w/v) each with pH 4.5 was subjected to batch fermentation. One litre of medium containing 20^{o}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 ^{o}C till no further decrease in ^{o}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. 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.

2.7 Maturation of wine

The clarified wine was kept for maturation. Ageing of both the wines namely, *Aloe*-amla and *Aloe*-ginger wine was done for one year in oak wood barrels (2.5 L), procured from M/S Jagatjit Industries Limited, Hamira, Punjab (India). The containers were filled upto the brim and analyzed for various components after one year of ageing.

2.8 Physico-chemical analysis of wine

TSS content was checked using a hand refractometer (Erma), pH was measured by digital pH meter, total sugars [23], titrable acidity [24], total soluble proteins [25], total phenolics [26], antioxidant activity [27] and ethyl alcohol [28] were determined by standard protocols. 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 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. Phytochemical screening of both the wines was also done qualitatively including tannins, terpenoids, flavonoids, saponins, glycosides, alkaloids, polysaccharides and amino acids by standard protocols [29, 30].

2.9 Sensory evaluation of wine

The organoleptic evaluation of matured *Aloe vera* based herbal wines was done by a panel of five judges on the basis of scoring in terms of appearance, colour, aroma, bouquet, acescent, total acid, sugar, body, flavour, astringency and general quality as per the prescribed performa [24].

2.10 *In vitro* antibacterial efficacy of *Aloe vera* based herbal wines against common food-borne pathogens

The antibacterial efficacy of prepared herbal 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.

2.11 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 [31]. 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* based herbal wine 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 herbal wines namely, *Aloe*-amla wine and *Aloe*-ginger wine. The plates were left for 30 min at room temperature for the diffusion of the test samples before being incubated at 37 ºC for 24 h after which the diameter of zones of inhibition were measured. The analyses were carried out in triplicates.

2.12 Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of *Aloe vera* based herbal wines

Minimum inhibitory concentration of wines for *S.* Typhimurium*, S. aureus, E. coli* was determined according to the method of Moretro and Daeschel, [9]. Both the prepared wines namely, *Aloe*-amla wine and *Aloe*-ginger 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 either of the test organism culture grown overnight and incubated for 24 h at 37ºC. 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 ºC 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.

2.13 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, [9] separately for both the wines. 10% (v/v) ethanol and *Aloe vera* based juices were used as controls. 50 µL of stationary phase (16 to 20 h) broth with approximately 10^9cfu/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 10^7cfu/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 ºC and bacterial count was taken. All analyses were carried out in triplicates.

2.14 *In-vitro* effect of *Aloe vera* based herbal wines against probiotic bacteria

The effect of *Aloe vera* based herbal 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 10^5cfu/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 ºC for 24 h. The diameters of the zones of inhibition if any, were measured after 24 h. All analyses were carried out in triplicates.

2.15 Effect of oral administration of *Aloe vera* based herbal wines on resident microflora of mice

The safety potential of *Aloe vera* based herbal 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 wines once in a day continuously for 3 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 prepared wines on the gut microflora. The feces were homogenized in normal saline, serially diluted and plated on MRS agar for the enumeration of lactobacilli [32]. The plates were incubated at 37 ºC for 24 h and the number of colonies appearing on the plates was recorded. All analyses were carried out in triplicates.

2.16 Statistical Analysis

All the results were expressed as mean±S.D. obtained from three trials. Comparisons were made by Student's t-test and $p < 0.05$ was considered significant.

3. Results and Discussion

The purpose of the study was to combine the useful properties of well known herbs with an aim to develop a new class of functional fermented *Aloe vera* based herbal wines and evaluation of their antimicrobial activity.

3.1 Production of *Aloe*-amla and *Aloe*-ginger wine

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. Both the substrates mixed separately with 50% *Aloe vera* gel (v/v) and supplemented with 0.1% (w/v) $(NH_4)_2HPO_4$, 0.1% (w/v) each of $MgSO_4.7H_2O$ and KH_2PO_4 along with cane sugar (TSS of 20ºB), pH 4.5, fermented at a temperature of 25 ºC provided a good substrate for *S. cerevisiae* to convert it into ethanol resulting in production of wine. The profiles of both the media studied in terms of pattern of pH, TSS, sugar

utilization and alcohol production at a regular interval of 24 h revealed the following results.

Sugar utilization in the *Aloe*-amla medium during the first 24 h was found to be 83.3mg/h/100mL. This picked up to 129.1mg/h/100mL and 208.3mg/h/100mL on 2nd and 3rdday, thereafter the rate of sugar utilization declined to 87.5mg/h/100mL, 83.3mg/h/100mL,79.1mg/h/100mL on the subsequent days and ultimately it declined at a constant rate of 62.5mg/h/100mL till the 10thday of fermentation period. On the other hand, the rate of alcohol production was 46.6µL/h/100mL during the first 24 h of incubation which increased gradually to 74.1µL/h/100mL during the next 24 h, further rising to 121.2µL/h/100mL during 3rdday. Highest rate of alcohol production was noted during the 3rdday of fermentation followed by gradual decline to 50.4µL/h/100mL, 44.1µL/h/100mL and ultimately it declined at a constant rate of to 30.8µL/h/100mL till the 10thday of fermentation period (Fig. 1). The pH of the medium revealed a gradual fall with the progress in fermentation, showing a final value of 3.7 at the end of fermentation. Quantitatively, the ethanol content in the wine, as observed after 10 days of fermentation, was 9.9% (v/v) with 88% fermentation efficiency.

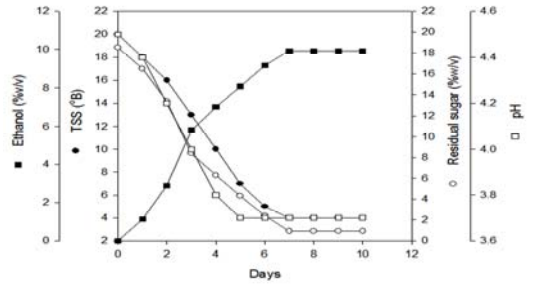

Fig 1: Pattern of sugar utilization, TSS, pH and alcohol production during the fermentation of *Aloe*-amla medium.

Sugar utilization in the *Aloe*-ginger medium during the first 24 h was found to be 83.3mg/h/100mL. This picked up to 166.6mg/h/100mL and 208.3mg/h/100mL on 2ndand 3rdday, thereafter the rate of sugar utilization declined to 83.3mg/h/100mL, 41.6mg/h/100mL, on the subsequent days and ultimately to 12.5mg/h/100mL till the 10thday of fermentation period. On the other hand, the rate of alcohol production was 47.08µL/h/100mL during the first 24 h of incubation which increased to 125.8µL/h/100mL on 2nd and 3rdday of fermentation period followed by gradual decline to73.7µL/h/100mL, 44.5µL/h/100mL, 20µL/h/100mL and ultimately to 7.08µL/h/100mL at the end of fermentation period (Fig. 2). The pH of the medium revealed a gradual fall with the progress in fermentation, showing a final value of 3.4 at the end of fermentation. Quantitatively, the ethanol content in the wine, as observed after 10 days of fermentation, was 9.9% (v/v) with 90% fermentation efficiency.

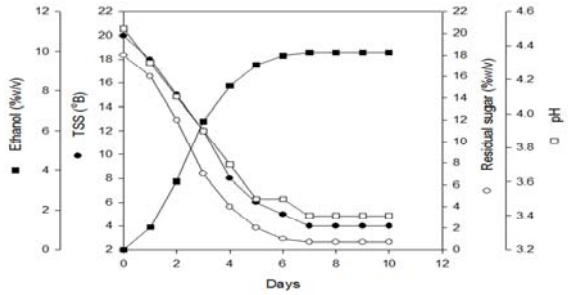

Fig 2: Pattern of sugar utilization, TSS, pH and alcohol production during the fermentation of *Aloe*-ginger medium.

3.2 Composition of matured *Aloe*-amla and *Aloe*-ginger wine

Aloe vera juice blended with hot water extract of amla and ginger proved to be a good growth medium for *S. cerevisiae* and got converted in to wine with the composition as depicted in Table 1. The prepared *Aloe vera* based herbal wines had a total soluble content between 4 to 4.6ºB with an average of 4.3ºB. For total acids the values quantified varied from 0.65to 0.71g/100 mL in equivalents of tartaric acid and the average value was 0.68g/100 mL in equivalents of tartaric acid with the pH of 3.7. The final total sugar content of wine ranges from 0.54 to 0.61% with an average of 0.57%. The ethanol content was found to be 9.9 % (v/v) for both the prepared wines.

Fermentation of supplemented *Aloe vera* based media brought the formation of various alcohols including methanol, n-propanol, n-butanol and iso-amyl alcohol the average levels of which, as observed in the resulting matured *Aloe*-amla and *Aloe*-ginger wines were 87.05 mg/L, 3.2 mg/L, 11.2 mg/L, and 62.8 mg/L respectively. These observations are agreeable to the earlier findings of several workers who also observed the formation of these alcohols during fermentation of fruit juices and reported that the proportion of these alcohols vary with the type of wine [3, 33].

The herbal wines prepared in this study exhibited the presence of esters detected in the form of ethyl acetate at an average level of 261.4 mg/L. The prepared wines showed the presence of Ca, Na, K as the major elements amounting to an average value of 516.0, 129.0, 405.9 mg/L respectively and the traces of Mg, Fe, Cu, Zn and Mn in the average range of 89.2, 401.5, 5.8, 85.0 and 8.6 µg/L respectively. The content of total phenols varied from 1218.6 mgGAE/L – 1797.05 mgGAE/L, averaging 1507.8 mg GAE/L, for the prepared herbal wines. The antioxidant activity assessed by determining the FRAP values varied from 3040.0 µmol/L to 4550.0 µmol/L averaging 3795.0 µmol/L for the prepared *Aloe*-amla and *Aloe*-ginger wine. These values are quite similar to the commercial wine values reported in the literature [34, 35].

Table 1 Composition of *Aloe vera* based herbal wines

Constituents	*Aloe*-amla wine	*Aloe*-ginger wine
Colour	Yellowish orange	Pale yellow
TSS (°B)	4.6±0.2	4.0±0.1
Total acids (g/100mL)	0.65±0.02	0.71±0.04
pH	3.7 ± 0.01	3.7 ± 0.02
Total sugar (g/100 mL)	0.61 ± 0.08	0.54 ± 0.05
Soluble proteins	1.6 ± 0.07	1.9 ± 0.08
Ethanol % (v/v)	9.9±1.12	9.9 ±1.19
Methanol (mg/L)	92.7±2.78	81.4±2.44
n-propanol (mg/L)	3.7±0.04	2.7±0.06
n-butanol (mg/L)	13.9±0.55	8.5±0.61
Iso – amyl alcohol (mg/L)	73.1±2.9	52.6±3.1
Ethyl acetate (mg/L)	258.6±10.34	264.3±11.02
Ca (mg/L)	520.0±12.34	512.0±11.31
Na (mg/L)	145.0±7.2	113.0±5.6
K (mg/L)	386.3±10.67	425.5±11.34
Mg (µg/L)	92.1±9.21	86.4±9.32
Fe (µg/L)	421.0±14.23	382.0±14.78
Cu (µg/L)	6.1±1.21	5.5±1.32
Zn (µg/L)	92.0±8.41	78.0±9.62
Mn (µg/L)	10.1±2.31	7.1±2.14
Phenolics (mgGAE/L)	1797.05 ± 32.88	1218.6 ± 11.99
Antioxidant activity (µmol/L)	4550.0±14.84	3040.0±17.07

Values are expressed as mean±SD of the observations (in triplicate) from three independent experiments.

The phytochemical profiling of *Aloe*-amla and *Aloe*-ginger wine revealed the presence of tannins, flavonoids, glycosides, polysaccharides and amino acids (Table 2). Bioactive

compounds usually found in herbs have been shown to possess potential health benefits with antibacterial, antioxidative, antihypertensive, anticarcinogenic and angiogenesis inhibitory activities [36-39]. Thus, the knowledge of presence of phytochemicals indeed proved to be helpful in assessing the quality of prepared wines and seems promising for exhibiting therapeutic effects.

Table 2 Phytochemical profile of *Aloe vera* based herbal wines

Phytochemical analysis	*Aloe*-amla wine	*Aloe*-ginger wine
Tannins	+	+
Flavonoids	+	+
Alkaloids	-	-
Saponins	-	-
Glycosides	+	+
Terpenoids	-	-
Polysaccarides	+	+
Free amino acids	+	+

3.3 Organoleptic evaluation of *Aloe vera* based herbal wines

The data pertaining to the evaluation for sensory quality of prepared *Aloe vera* based herbal wines namely, *Aloe*-amla and *Aloe*-ginger is presented in Table 3 and 4 respectively. The acceptability of the prepared wines was ascertained by a panel of 5 judges. The mean score of sensory evaluation by the five judges were found to be 18.4, 17.2 for the matured *Aloe*-amla wine, *Aloe*-ginger wine respectively. Both the prepared wines were acceptable and observed to possess outstanding sensory characteristics as revealed by the organoleptic scores given by the panel of judges.

Table 3 Evaluation card of *Aloe*-amla wine by five tasters

Characteristic	Max. score	Score by tasters				
		I	II	III	IV	V
Appearance	2.0	2.0	2.0	2.0	2.0	2.0
Colour	2.0	2.0	1.5	2.0	2.0	2.0
Aroma	2.0	1.5	2.0	1.0	2.0	2.0
Bouquet	2.0	2.0	2.0	2.0	2.0	2.0
Acescent	2.0	2.0	1.5	2.0	2.0	1.0
Total acid	2.0	2.0	2.0	2.0	1.0	1.5
Sugar	1.0	0.5	1.0	0.5	1.0	1.0
Body	1.0	1.0	1.0	1.0	1.0	1.0
Flavour	2.0	2.0	1.5	2.0	1.0	2.0
Astringency	2.0	2.0	2.0	2.0	2.0	1.5
General quality	2.0	2.0	2.0	2.0	2.0	2.0
Total	**20.0**	**19.0**	**18.5**	**18.5**	**18.0**	**18.0**

Table 4 Evaluation card of *Aloe*-ginger wine by five tasters

Characteristic	Max. score	Score by tasters				
		I	II	III	IV	V
Appearance	2.0	1.5	1.5	2.0	2.0	2.0
Colour	2.0	1.0	1.0	1.5	1.0	1.0
Aroma	2.0	2.0	2.0	2.0	2.0	2.0
Bouquet	2.0	2.0	2.0	2.0	2.0	2.0
Acescent	2.0	2.0	1.5	2.0	2.0	1.0
Total acid	2.0	2.0	2.0	2.0	1.0	1.5
Sugar	1.0	0.5	0.5	0.5	1.0	0.5
Body	1.0	1.0	1.0	1.0	1.0	1.0
Flavour	2.0	2.0	2.0	2.0	2.0	2.0
Astringency	2.0	1.0	1.0	2.0	1.0	1.5
General quality	2.0	2.0	2.0	2.0	2.0	2.0
Total	**20.0**	**17.0**	**16.5**	**19.0**	**17.0**	**16.5**

3.4 *In vitro* antibacterial efficacy of *Aloe vera* based herbal wines against common food borne pathogens

The antibacterial efficacy of prepared wines against *S.* Typhimurium, *S. aureus* and *E. coli,* the common food-borne pathogens, was assessed by the presence or absence of inhibition zones, MIC and MBC values and time kill curves.

3.4.1 Zone of inhibition by well diffusion assay

To investigate the antibacterial efficacy of the *Aloe vera* based herbal wines against known pathogenic organisms like *S.* Typhimurium, *S. aureus* and *E. coli*, bacterial growth inhibition zones were observed for both the wines by well diffusion assay. Table 5 shows the recorded size of zone of inhibition against the pathogens by prepared wines and their controls. It was observed that both the wines showed remarkably higher efficacy of inhibition in comparison to respective controls like 10% ethanol and herbal extracts. Average zone sizes with *Aloe*-amla and *Aloe*-ginger wine against the three organisms measured about 12.3 and 11.0 mm respectively making *Aloe*-amla wine slightly more efficacious in terms of its antibacterial activity as compared to its counterpart. *Aloe*-amla wine had the highest antibacterial activity against *S.* Typhimurium and *E. coli* while *Aloe*-ginger wine worked best against *S. aureus*. Whereas, 10% ethanol alone accounted for an average zone size of 2.7 mm, other components like *Aloe*-amla and *Aloe*-ginger extract measured 3.7 and 3.3 mm respectively. The data signifies that the synergistic effect of all components of wine results in a drastically improved antimicrobial potential as the unfermented herbal extracts were far weaker to inhibit bacterial growth. This observation is well supported by the work of several authors who have noted that wine has a greater antibacterial effect compared with the same concentration of diluted absolute ethanol and grape juice [7, 40, 41]. The antimicrobial activity has been studied for ethanol, methanol and acetone extracts of *Aloe vera* gel powder against four gram-negative (*Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*) and gram-positive (*Bacillus cereus*, *Bacillus subtilis*, *Streptococcus pyogenes, Staphylococcus aureus*) bacteria using the agar well diffusion method [42]. The methanol and ethanol extracts established higher activity than the acetone extract against most of the tested pathogens (the first two had an inhibition zone ranging between 12.6 and 23.3mm and the maximum value was obtained for *Bacillus cereus*). However, the inhibition zone achieved on using the acetone extract ranged from 6.0 (for *Escherichia coli*) to 7.3mm (for *Streptococcus pyogenes*) and activity was not detected for *Pseudomonas aeruginosa* and *Salmonella typhi*. Generally, these extracts showed better activity against gram-positive bacteria [42]. In our study, both the variants of *Aloe vera* based herbal wines showed comparable antibacterial efficacy against the tested food-borne pathogens as recorded by the size of zone of inhibition (Table 5).

Table 5 Size of Zone of Inhibition (mm)

Sample	Zone of inhibition (mm)		
	S. Typhimirium	*S. aureus*	*E. coli*
Aloe-amla extract	4.0±0.06	3.0 ±0.06	4.1±0.07
Aloe-ginger extract	2.7±0.05	4.0±0.04	3.2±0.08
10% ethanol (v/v)	2.5±0.06	2.9±0.05	2.7±0.05
Aloe-amla wine	14.0±0.09	11.0±0.07	12.0±0.07
Aloe-ginger wine	10.5±0.11	11.5±0.08	11.0±0.14

Values are expressed as mean±SD of the three different observations.

3.4.2 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of *Aloe vera* based herbal wines

Minimum inhibitory concentration (MIC) for both the wines was determined against each of the three pathogenic bacteria including *S.* Typhimurium, *S. aureus* and *E. coli*. MIC for *S.* Typhimiurum was found to be 25% and 40% for *Aloe*-amla and *Aloe*-ginger wine respectively. For *S. aureus,* MIC was

recorded as 40% and 30% for *Aloe*-amla wine and *Aloe*-ginger wine respectively. MIC of *Aloe*-ginger wine was 50% whereas MIC for *Aloe*-amla wine was found to be 40% against *E. coli*. Thus, *Aloe*-amla wine exhibited the highest efficacy against *S.* Typhimurium and *E. coli* while for *S. aureus Aloe*-ginger wine worked the best. Minimum bactericidal concentrations (MBCs) of the wines against the three test pathogens were determined by plating the contents of the tubes containing MICs and higher concentrations of the wines on nutrient agar and incubated at 37 ºC for 24 h. The MBC of *Aloe*-amla wine was found to be 35% for *S.* Typhimurium and 45% for both *E. coli* and *S. aureus*. MBC of *Aloe*-ginger wine was more than 50% for *S.* Typhimurium and *E. coli* whereas for *S. aureus* it was found to be 40% (Table 6). The antibacterial activity of *Aloe vera* inner gel has been tested in gram-positive and gram-negative bacteria by several methods. [43] Freeze dried *Aloe gel* powder extracts have been evaluated in diverse solvents such us hexane, chloroform, methanol and water against gram positive (*Staphylococcus aureus*, *Enterococcus bovis*) and gram-negative (*Shigella flexneri*, *Enterobacter cloacae*) bacteria. These experiments disclosed lower MIC values for *Aloe vera* gel than for the aqueous extract [44].

Table 6 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of *Aloe vera* wines against selected pathogens.

Pathogen	Wine	MIC	MBC
S. Typhimurium	*Aloe*-amla wine	25%	35%
	Aloe-ginger wine	40%	> 50%
S. aureus	*Aloe*-amla wine	40%	45%
	Aloe-ginger wine	30%	40%
E. coli	*Aloe*-amla wine	40%	45%
	Aloe-ginger wine	50%	> 50%

Values are based on results from 3 parallel experiments. The wines were tested at 5% interval.

3.4.3 Time dependent bactericidal assay

The *Aloe vera* based herbal wines tested were found to be strongly effective against *S.* Typhimurium, *S. aureus* and *E. coli*. Exposure to wines decreased the bacterial count of all the three pathogens from 10^7cfu/mL to undetectable levels within 20 min as shown in Fig. 3, 4, 5. Time kill curve of the three pathogenic organisms was observed at five different time intervals i.e. 0, 5, 10, 15, 20 min after exposure to both the wines and corresponding unfermented herbal extracts, 10% ethanol as controls separately. An initial count of nearly 10^7cfu/mL was used for the three pathogens. Both the tested wines displayed a remarkable antimicrobial response against the pathogens. All three pathogens were completely inactivated after 20 min of exposure to each wine.

S. Typhimurium and *E. coli* were completely inactivated in 15 min by *Aloe*-amla wine while *Aloe*-ginger wine took 20 min for the same. Nearly 2 log reduction in growth was observed in the first five minutes for both the wine variants. Again, the respective controls showed relatively much weaker antibacterial activity with an average one log reduction in growth even after 20 min of exposure.

In case of *S. aureus*, *Aloe*-ginger and *Aloe*-amla wines were most successful as these eliminated the pathogen after 15 min of exposure. Here again, *Aloe*-ginger wine was slightly more effective in comparison to *Aloe*-amla wine. Synergism of all individual components in wines was probably responsible for the strong antimicrobial potential even in this case as the respective controls could only reduce the organism by one log after 20 min of exposure.

Our results are well supported by various studies like Weisse *et al*. [7] who reported 5-6 log cycles reduction in the viable numbers of *S. enteritidis, S. sonnei* and *E. coli* in 20 min of exposure time with wine. Similar inactivation patterns were shown by Sugita-Konishi *et al*. [8] for *S. enteritidis*, *E. coli* O157:H7 and *V. parahaemolyticus*. The same extent of inactivation was attained for *Salmonella* spp. and *E. coli* in 5-30 min and 20-60 min, respectively [41, 45]. Carneiro *et al*. [46] reported that the bacterial count of 10^6–10^7cfu/mL exposed to wine were evidently inactivated to undetectable numbers within 30 sec. indicating the strong bactericidal effect of wine on the survival of the tested strains of *C. jejuni*. Moretro and Daeschel, [9] while determining the effectiveness of red and white wine against various strains of *S. aureus*, *L. monocytogenes*, *E. coli* O157:H and *S.* Typhimurium reported a reduction of 6 log cycles after 10 min of exposure in case of *S.* Typhimurium.

A number of studies have established the effectiveness of wine against a range of food-borne pathogens. The results presented in the above experiments assessing the *in vitro* antibacterial efficacy of the prepared *Aloe vera* based herbal wines suggests that the studied food-borne pathogens are sensitive to the exposure to both *Aloe*-amla as well as *Aloe*-ginger wine. Ethanol, organic acids, polyphenols and low pH, are the various constituents of wine known to contribute to its antibacterial potential. Reported studies in the literature supporting the antibacterial potential of wine are normally grouped into two categories; those studying the importance of wine phenolics and other giving prominence to the non-phenolic constituents of wine. Daglia *et al*. [47], reported organic acids in wine to be responsible for the displayed antibacterial actions against oral streptococci while the extracted polyphenols exhibited no activity against the microorganisms. Further, they tested dealcoholized red (DRW) and white (DWW) wines against a range of *Streptococcus pyogenes* and oral streptococci. The reported MICs and MBCs obtained from the dealcoholized wines showed that both wines are active and that the difference in antibacterial activities are strain dependent. DRW was found to have a lower minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) than DWW, with MIC for the DRW ranging from 10-20% volume of wine, and ranging from 20-30% in volume for the DWW. The MBC for the DRW ranged from 20-40% volume and from 30-50% volume for the DWW. Carneiro *et al*. [46] reported that ethanol and certain organic acids act synergistically and represent the major components for the bactericidal effects of wine on *Campylobacter*. In a related study, ethanol or water solutions of pH values corresponding to those of wine showed minor activity against *Salmonella enteritidis* when applied separately [40]. Moretro and Daeschel, [9] found that the combination of organic acids (malic and tartaric) with ethanol (15%) and low pH (≤ 3.0) had significantly stronger antimicrobial activity than the sum of the individual effects of these components against various food-borne pathogens, indicating potential synergistic interactions. Waite and Daeschel, [48] reported pH to be the critical factor in predicting inactivation of tested pathogens while examining four different wine parameters ie., pH, ethanol, titratable acidity and sulphur dioxide in various combinations. In the study by Just and Daeschel, [41] ethanol was found to be responsible for the displayed antimicrobial activity against *E. coli* O157:H7 and *Salmonella* spp., as wine when compared to grape juice demonstrated improved antimicrobial activity. Ethanol causes changes in cell membrane permeability which may lead to enhanced efficacy of organic acids and may partly explain the reason for the improved antimicrobial activity of wine relative to grape juice [41, 45, 49]. Thus, the antibacterial

potential of wine may be the result of synergism among organic acids, ethanol and low pH.

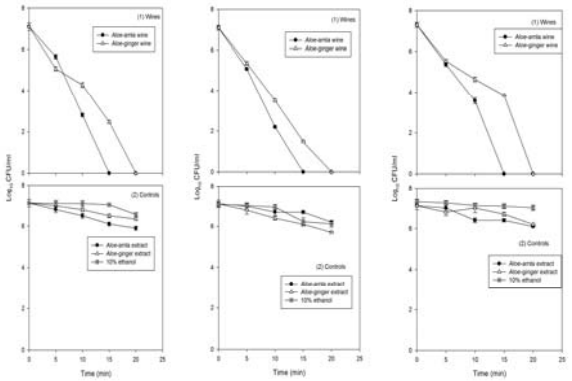

Fig 3, 4, 5: The pattern of reduction of *S.* Typhimurium, *S. aureus, E. coli* counts in presence of both the *Aloe vera* based herbal wines (1) and their respective unfermented extracts and 10% (v/v) ethanol (2), indicating bactericidal activity. The data represents mean ± SD of three independent experiments.

In contrast to the above-mentioned studies, there are substantial numbers of reports evaluating the role of phenolic compounds for the antibacterial potential of wine. Radovanovic *et al*. [50] reported total phenolic and monomeric anthocyanin content of wines to be responsible for the antimicrobial activity. In a study by, Vaquero *et al*. [51] the antimicrobial effects of polyphenolic compounds present in red wines was observed as the antimicrobial properties of tested wines were found to improve with the increase in their polyphenols content. Furthermore, several studies ascertained the antibacterial potential of different isolated phenolic compounds, which are normal constituents of wine [52-54]. Inhibition of DNA gyrase, inhibition of cytoplasmic membrane function and energy metabolism are the proposed mechanisms responsible for the antibacterial activity of polyphenols [55]. Boban *et al*. [56] specified that antimicrobial potential of wine cannot be exclusively credited to its phenolic or non-phenolic components. In relation to separate the role of wine phenolics, ethanol, and pH from other wine components, the antimicrobial activity of intact wine against *Salmonella enterica* serovar Enteritidis and *Escherichia coli* were compared to that of phenols-stripped wine, dealcoholized wine, ethanol, and low pH applied separately and in combination. The array of antibacterial efficacy of the test samples was found to be: intact wine > phenols-stripped wine > dealcoholized wine > combination of ethanol and low pH > low pH > ethanol which confirms the synergistic effect of wine that contributes to the antibacterial value of wine. Although the contribution of individual wine components (ethanol, organic acid, low pH and phenolic compounds) to the antibacterial effect of wine on the test organisms were not studied in the present study but it can be assumed that the synergistic action of different wine components and the bioactive components present in the substrates used for the production of herbal wines may be responsible for the displayed bactericidal effect.

3.5 *In-vitro* effect of *Aloe vera* based herbal wines against probiotic strain and effect of oral administration of wines on resident microflora of mice

Probiotics as an adjunct to other therapies has been developed as a new therapy in the treatment of various bacterial gastrointestinal infections [57]. Probiotic strains, were exposed to both the herbal wines and the same were observed to survive in the presence of wines. *Aloe vera* based herbal wines were found to be safe towards the probiotic strains including *L. casei*, *L. plantarum* and *L. acidophilus*, which are important in the gut of the animal and the same were not inhibited. These bacteria continued to grow in the presence of wine without any discernible zone of inhibition around each well. Furthermore, there was no significant difference in viable count of lactobacilli in the feces of mice fed with wines for a period of 3 weeks (Table 7). Other studies have also found that wine provides protection against food-borne pathogens and alongside provides digestive benefits and intestinal health by promoting the propagation of friendly bacteria [58, 59]. Wine consumption was found to increase the levels of gut probiotics which are important to digestive health and protect against various other systemic infections [60]. It is the polyphenols in wine that promote the growth of probiotic bacteria and inhibit non-beneficial bacteria from the human microbiota, encouraging health benefits in the host [61].

Table 7 Viable count of lactobacilli in feces of wine fed mice

Days	Fecal count (Log_{10} cfu/g faeces[a])	
	Aloe-amla wine fed	*Aloe*-ginger wine fed
0 Day	7.02±0.21	7.1±0.25
1st week	7.2±0.31	7.5±0.34
2nd week	7.1±0.18	7.2±0.12
3rd week	7.3±0.12	7.9±0.14

[a]Values are expressed as mean±SD of the three different observations.

4. Conclusions

The current work was aimed at exploring the vast alternatives of beneficial herbs and botanical ingredients to further enhance the efficacy and functionality of ever popular health beverage i.e. wine. The work was carried out to develop process methodology for the production of *Aloe*-amla and *Aloe*-ginger wine. Furthermore, the newly developed variants were assessed for the antibacterial potential. The prepared *Aloe vera* based herbal wines were found to possess bactericidal effect against common food-borne pathogens, the bad bacteria and no harmful effect against tested probiotic strains, the good bacteria. 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. Further studies have been planned to assess the therapeutic potential of the prepared wines in a suitable murine model.

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