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Summary and Conclusion
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SUMMARY AND CONCLUSIONS

Wine is one of the highly acceptable class of beverages as it serves as a vital adjunct to the human diet and is now increasingly being recognized for conferring various health benefits due to its antioxidant potential. *Aloe vera* along with amla, ginger and red grapes known for their nutraceutical properties were used as the substrates for the production of different variants of *Aloe vera* wine and evaluated for enhancement of their therapeutic potential. The present work entitled “PROCESS DEVELOPMENT FOR THE PRODUCTION OF VALUE ADDED HERBAL WINES FROM *Aloe vera* AND EVALUATION OF THEIR THERAPEUTIC POTENTIAL” deals with the standardization of process methodologies for the production of *Aloe vera* based herbal wines. These wines were further evaluated for the antibacterial and antioxidative potential that these were assumed to confer in a *Salmonella* infected murine model. The major findings of the present study are:

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

   *Aloe vera* gel was extracted from fresh leaves, blended to homogeneity and supplemented with cane sugar to a TSS of 20°B to be used as a medium for the growth of *S. cerevisiae* to convert it into ethanol. After 10 days of fermentation, observed ethanol content was 5.3% (v/v) with 70% fermentation efficiency.

2. **Standardization of various process parameters for the production of *Aloe vera* wine**

   The process parameters for the production of *Aloe vera* wine were standardized by varying various environmental and nutritional factors, one at a time. The effect of environmental factors was studied by varying the incubation temperature, inoculum level, pH of the medium, while the effect of nutritional factors was evaluated by varying the sugar concentration, supplementing various nitrogen sources and metal salts in the *Aloe vera* based media. Of the various environmental factors, an incubation temperature of 25°C, 10%
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inoculum and a pH of 4.5 resulted in maximum production of ethanol. Amongst the nutritional factors, a sugar concentration of 20°B, nitrogen source in the form of (NH₄)₂HPO₄, metal salts like MgSO₄.7H₂O and KH₂PO₄ resulted in highest fermentation efficiencies and thus the ethanol yields.

3. Production of Aloe vera wine under optimized conditions

Production of Aloe vera wine under standardized environmental and nutritional conditions resulted in highest fermentation efficiency of 90%, yielding a clear pale yellow Aloe vera wine with 10% (v/v) alcohol. The levels of other constituents like total acids, other alcohols, esters, minerals, phenolics, antioxidant activity in the prepared wine were quite similar to the components found in various commercial wines. The aging of Aloe vera wine in oak wood barrel, as attempted in the present study, improved the characteristics in terms of clarity, colour and proved to be very useful in bringing down the levels of other alcohols including methanol, n-propanol, n-butanol, iso-amyl alcohol to 112.8±3.32, 1.3±0.07, 10.6±0.53, 39.5±0.70 mg/L respectively. It brought about an increase in ethyl acetate and total phenolic content which rose to the levels of 351.3±1.96 mg/L and 1065.0±10.11 mg GAE/L respectively. There was a significant increase in the antioxidant activity of the matured wine sample to 2950.0±18.48 μmol/L relative to 1132.0±7.2 μmol/L in the unmatured Aloe vera wine sample signifying the importance of maturation. Maturation also led to an improvement in the aroma and organoleptic characteristics as the matured Aloe vera wine was adjudged to be better after tasting by a panel of five judges when compared to the unmatured counterpart. Phytochemical profiling of Aloe vera wine revealed the presence of medicinally active constituents including tannins, flavonoids, glycosides, polysaccharides, free amino acids. However, the matured Aloe vera wine sample revealed the presence of same phytochemicals as present in the unmatured counterpart.

4. Assessment of safety of prepared Aloe vera wine

The safety of prepared Aloe vera wine was assessed in a mice model by measuring important parameters of liver and kidney function and the histological alteration after oral administration of Aloe vera wine for a period of four weeks. No significant difference was observed in the values of various renal and liver function test indicators at day 10 and 20
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when compared with control group values (0 day) by administration of wine. Moreover, Aloe vera wine consumption appeared to have no adverse effects on the mouse liver, as revealed by the histology of the liver section from the wine fed group as no histological damage was seen and the overall histology was same as that of the control group which was not fed with wine. This indicated that Aloe vera wine fed for a period of 4 weeks had no harmful effect and thus appears to be safe for oral consumption.

5. Production of different variants of Aloe vera wines

In order to add more value to the product and add new varieties in the category of herbal wines different variants were prepared by supplementing the Aloe vera juice with additional herbs / fruits including amla, ginger and red grapes. Aloe vera juice blended with hot water extract of amla, ginger and grape juice also proved to be a good growth medium for S. cerevisiae and got converted in to wine with ethanol content ranging from 9.9% (v/v) to 10.1% (v/v) with an average of 10.02% (v/v). The values of other constituents like total acids, other alcohols, esters, minerals, phenolics, antioxidant activity etc. determined in the prepared wines were quite similar to the commercial wine values reported in the literature. Furthermore, phytochemical profiling of Aloe vera wine samples revealed the presence of medicinally active constituents including tannins, flavonoids, glycosides, polysaccharides, free amino acids. The content of total phenols varied from 800.0 mgGAE/L – 1797.0 mgGAE/L, averaging 1305.0 mgGAE/L, for the four prepared Aloe vera wines. The highest concentration was detected in Aloe-amla wine followed by Aloe-red grape wine, Aloe-ginger wine and Aloe vera wine. Antioxidant capacity was measured according to the FRAP method. All analyzed wines demonstrated significant reducing capacity as evident from FRAP values of the wines which ranged from 2950.0 μmol/L-4550.0 μmol/L, averaging around 3452.0 μmol/L signifying a substantial antioxidant capacity of these wines. Thus, the antioxidant activity of the wines studied correlated well with their total phenolic content.

6. Organoleptic evaluation of prepared wines

The sensory parameters of the prepared wines were ascertained by a panel of 5 judges. Organoleptic scores of the matured variants of Aloe vera wine prepared in the present study revealed that the herbal wines are acceptable as all the variants were found to possess outstanding sensory characteristics.
7. **In-vitro antibacterial efficacy of Aloe vera wines**

The antimicrobial activities of prepared wines against *S. Typhimurium*, *E. coli* and *S. aureus* were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, MIC and MBC values, time kill curves.

a) The zone of inhibition of all the wines was measured against *S. Typhimurium*, *S. aureus* and *E. coli*. It was observed that all the four wines showed remarkably higher efficacy of inhibition in comparison to respective controls like 10% ethanol and herbal extracts. Average zone sizes with *Aloe vera*, *Aloe-amla*, *Aloe-ginger* and *Aloe-red grape* wines against the three organisms measured about 10.0, 12.5, 11.0, 10.5 mm respectively making *Aloe-amla* wine slightly more efficacious in terms of its antibacterial activity as compared to its counterparts. *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 vera* extract, *Aloe-amla*, *Aloe-ginger*, *Aloe-red grape* extract measured 2.2, 3.5, 3.3 and 2.9 mm respectively.

b) In consistence to the above data, *Aloe-amla* wine showed highest efficacy against *S. Typhimurium* and *E. coli* with MIC of 25% and 40% respectively. *Aloe-ginger* wine worked the best against, *S. aureus*, with a MIC of 30%. All the wines were able to effectively curb the three tested pathogens with MIC ranging from 25% to 50%. 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°C for 24 h. The MBC of *Aloe vera* wine was 40%, 50% and more than 50% for *S. Typhimurium*, *S. aureus* and *E. coli* respectively. MBC of *Aloe-amla* wine was found to be 35% for *S. Typhimurium* and 45% for *E. coli* and *S. aureus*. MBC of *Aloe-ginger* wine and *Aloe-red grape* wine was more than 50% for *S. Typhimurium* and *E. coli* whereas for *S. aureus* it was found to be 40% and 50% respectively.
c) 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 all wine variants and corresponding unfermented herbal extracts, 10% ethanol as controls separately. An initial count of nearly $10^7$ cfu/mL was used for the three pathogens. All the tested wines displayed a remarkable antimicrobial response for the pathogens. All three pathogens were completely inactivated after 20 min of exposure to each wine.

8. **In-vitro effect of Aloe vera wines against probiotic bacteria and effect of oral administration of Aloe vera wines on resident microflora of mice**

Aloe vera wines were found to be safe towards the probiotic strains including *L. casei*, *L. plantarum* and *L. acidophilus*, which are important microflora of the gut as the 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.

9. **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, histological analysis, liver markers. The study was carried out in a murine experimental model comprising of four groups namely, Control, Wine *per se* group, *Salmonella* infected and Infected-wine fed, each comprising of at least 10 mice for the prepared herbal wines.

a) **Bacterial translocation in the liver, spleen and intestine**

The bactericidal effect of ingested wine on *S. Typhimurium* was studied in a murine model. Recovery of *S. Typhimurium* was seen in the homogenates of the *Salmonella*-challenged group. A 2-3 log fold decrease in the bacterial load in liver, spleen and small intestine of the infected-wine fed animals was observed relative to infected control thus suggesting the therapeutic activity of wines against *S. Typhimurium* infection.
b) Effect of administration of Aloe vera wines on liver damage caused due to Salmonellosis

S. Typhimurium infected group revealed marked pathological changes such as kupffer cell hyperplasia, necrosis and a significant liver cell nucleus enlargement. In contrast, the histological examination of tissue sections from the infected wine-fed group showed an improvement of liver morphology with comparatively mild kupffer cell hyperplasia, absence of necrotic cells, restoration of morphology of liver cells.

10. Evaluation of liver function by assessing serum ALT and AST activity following wine administration

In the present study, significant increase of both ALT and AST levels was observed in infected mice as compared to control which provided evidence in support to damage to liver. However, administration of herbal wines to the infected groups restored activity of these serum enzymes comparable to the control group. No negative effects were observed on the marker enzymes of hepatic damage in the wine-fed per se groups with respect to control.

11. Antioxidative potential of the prepared Aloe vera wines

The proposed antioxidative potential of the prepared wines was estimated by measuring malondialdehyde (MDA) levels, superoxide dismutase (SOD) levels and glutathione (GSH) content.

i) Effect on peroxidative damage in liver

In the present study, effect of wine administration on oxidative stress generated during murine Salmonellosis was studied. It was observed that Salmonella infection produces significant oxidative stress as the levels of MDA increased significantly in the mice challenged with S. Typhimurium as compared to the control animals receiving normal (p<0.001). Wine administration had a healing influence on the generated oxidative stress as infected-wine fed groups displayed reduced lipid peroxidation as compared to the infected counterpart. The maximal effect was observed with Aloe-ama wine which showed 56.01% decrease in MDA levels as compared to infected control. All the other three wines ie., Aloe vera, Aloe-ginger, Aloe-red grape wine also significantly reduced the MDA levels (p<0.001).
ii) Analysis of hepatic antioxidant profile in terms of superoxide dismutase (SOD) levels and reduced glutathione (GSH) levels

The presence of oxidative stress produced due to *Salmonella* infection was inferred from the depletion of antioxidant GSH content and reduction in the activity of SOD in liver tissues of infected mice. The administration of wine strongly ameliorated the capacity of liver to metabolize ROS, as shown by increased SOD and GSH levels. *Aloe*-amla wine showed maximum increase of (39.7%) followed by *Aloe*-red grape wine (36.4%), *Aloe vera* wine (31.9%) and *Aloe*-ginger wine (20.1%) in SOD activity compared to infected control. Moreover, wine administration resulted in higher hepatic superoxide dismutase activity per se groups compared to the control. Furthermore, the reduced glutathione levels were increased by the administration of *Aloe vera*, *Aloe*-amla, *Aloe*-ginger and *Aloe*-red grape wine by 29.9%, 41.8%, 34.5%, 41.0% respectively, in the infected-wine fed groups as compared to infected control. Per se groups showed fairly significant change in the levels of GSH (p<0.05) when compared to control.

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

The antioxidant defense system appears to be enhanced in the plasma, by increased FRAP levels in the experimental groups. As compared with the control group, FRAP values were markedly decreased in infected control, but it increased by the administration of *Aloe vera* wine, *Aloe*-amla wine, *Aloe*-ginger wine and *Aloe*-red grape wine by 41.1%, 51.6%, 48.2% and 53.8% respectively, in the infected-wine fed group reaching significance between both groups (p<0.001). Moreover, there was a significant increase of an average 30% in the FRAP values in per se groups as compared to control (p<0.001) which is suggestive of an improved antioxidant status in the wine fed mice.

13. Morphological changes in the cells of *S. Typhimurium* in the presence of *Aloe vera* wine

Scanning electron microscopy (SEM) provided qualitative information of the change in the morphology of *S. Typhimurium* treated with *Aloe vera* wine. SEM observation of *S. Typhimurium* treated with 50% *Aloe vera* wine for 6 h revealed that the treated bacteria were much shorter and their outer membrane was rough and deformed as compared to the
untreated ones. SEM examination of *S. Typhimurium* treated with 50% *Aloe vera* wine for 24 h revealed membrane disruption that appeared to have caused leakage of intracellular contents.

In conclusion, 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 study has yielded a variety of *Aloe vera, Aloe-amla, Aloe-ginger* and *Aloe-red grape* based herbal wines with bactericidal effect against common food-borne pathogens, the bad bacteria and no harmful effect against tested probiotic strains, the good bacteria. The wines exerted a therapeutic and protective efficacy against *Salmonella* infection in a murine model. The wine feeding effectively decreased the bacterial load in liver, spleen and small intestine of the infected-wine fed animals relative to infected control thus, suggesting the therapeutic activity of wines against *S. Typhimurium* infection. Wine administration exerted a protective efficacy against the generated oxidative stress by reducing lipid peroxidation and increasing the levels of hepatic superoxide dismutase, reduced glutathione and plasma antioxidant capacity. It is suggested that the *Aloe vera* wines, as prepared in the present study, can be regularly consumed for increasing the antioxidant status and to decreases oxidative stress in the circulation. The *Aloe vera* wine consumption may also be used as an adjunct therapy to combat Salmonellosis and to keep the probiotic strains in healthy state. The investigation encourages further studies to isolate the active compounds from these wines that are responsible for the hepatoprotective effects, the mechanism of action involved in the antioxidant effect and translocation of pathogenic bacteria from infected tissues. More efforts need to be directed towards scientific evaluation for their efficacy by subjecting to vigorous pre-clinical studies followed by clinical trials to unravel the mysteries hidden in the prepared herbal wines.