CHAPTER-3

Materials and Methods

3.1. Materials

3.1.1. Microorganism

Saccharomyces cerevisiae HT was already available in our lab and was used in study.

3.1.2. Glassware and plasticware

Glassware from Borosil and Schott Duran; plasticware from Tarsons and Laxbro were used after washing with the detergent and rinsing in distilled water. They were then dried in hot air oven at 70°C before use.

3.1.3. Chemicals and reagents

Streptozotocin (STZ) was purchased from Sigma Chemical Co. (St. Louis, USA). Glucose-oxidase peroxidase glucometer was obtained from Abbott India Ltd. for analysing blood glucose levels. Huminsulin (Eli Lilly and Company (India Pvt. Ltd.) and Glibenclamide (Daonil), were procured from a local chemist. Red wine (Tocornal Merlot 2011) was received as a generous gift from Mr. Gaurav Rose. All the other 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).

Bio-chemical assays for total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) and serum Insulin were performed using colorimetric kits obtained from Reckon India Pvt. Ltd and Crystal Chem (US).

Hepatic and renal function analysis was done by the estimating the levels alanine transaminase (ALT), aspartate transaminase (AST), alkaline ahosphatase (ALP), creatinine, urea and uric acid in serum with the help of commercially available kits obtained from Reckon India Pvt. Ltd, Erba Mannheim, Germnay and Accurex Biomedical Pvt. Ltd.

3.1.4. Culture media

3.1.4.1. Glucose yeast extract (GYE) broth

Glucose	1.0 g
Malt extract	3.0 g
Yeast extract	3.0 g
Peptone	5.0 g
Distilled water	1000 ml

The above components were dissolved in 900 ml of distilled water and thereafter, pH was adjusted to 4.5 followed by making the final volume to 1000 ml with distilled water and the broth was then autoclaved at 15 psi for 15 min.

3.1.4.2 Potato dextrose agar (PDA)

Potatoes infusion	200.0 g
Dextrose	20.0 g
Agar	30.0 g
Distilled water	1000 ml

The above components were dissolved in 1000 ml distilled water and pH was adjusted to 4.5 and thereafter, addition of (3.0%) agar, followed by autoclaving at 15 psi for 15 min.

3.1.5. Raw substrates for wine production

Syzygium cumini leaves and stem were collected from Panjab University campus, Chandigarh and the fruits were purchased from the local market of Chandigarh city. These were washed in clean water and dried in air. The plant was identified by Prof. Promila Pathak and a specimen of the plant was deposited in the herbarium of Department of Botany, Panjab University, Chandigarh. The specimen number assigned was 21045.

3.1.6. Animal feed

Rat feed was obtained from (M/s. Ashirwad Industries Pvt. Ltd., Punjab, India). All the animals were fed with the standard pellet diet comprising of 20-21% crude protein, 4% fat, 5.0-5.75% crude fiber, 8-9% ash, 1.0-1.5% calcium, 0.6-0.8% phosphorus and 50% nitrogen free extract.

3.1.7. Animals and Ethics

Male wistar rats (200–250 g) were procured from Central Animal House, Panjab University, Chandigarh (India). The animals were housed under standard laboratory

conditions, maintained on a 12:12 h light:dark cycle. These were housed in polypropylene cages (5 rats per cage) bedded with clean rice husk in well-aerated animal room of the Department of Microbiology. All the animals had free access to the feed 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 (approval ID: PU/IAEC/F/15/84, dated 15/9/2015) 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.

3.2. METHODS

3.2.1. Maintenance of yeast

S. cerevisiae was maintained on PDA slants prepared as per the method described in section 3.1.4.2. The slants were stored at 4°C and were sub-cultured every month. For long term storage, glycerol stocks were prepared and kept at -70°C. Before use, the organism was sub-cultured in respective broth, followed by plating and then isolated colony was picked up for further use.

3.2.2. Process development for the fermentation of *Syzygium cumini*

The process for fermenting different parts of *S. cumini* involved the following steps:

3.2.2.1. Processing and extract preparation of *S. cumini* stem, leaves, seed and pulp

Jamun berries were procured from local market of Chandigarh. Berries were boiled for 20 minutes and deseeded mechanically by hands. The pulp was crushed in a blender and water was added to the must obtained in a ratio of 1:1. This was then used for further experiments. Jamun leaves and stem were obtained from Herbal Garden, Panjab University, Chandigarh. The seeds, leaf and stem were washed properly and added separately to DW containing flasks at a ratio of 15 (% w/v). They were steamed at 5 psi for 20 minutes in a laboratory autoclave and then used for further experiments.

3.2.2.2. Inoculum preparation

25 mL of sterilized GYE broth, prepared as per section 3.1.4.1 was 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 for 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 Jamun pulp/leaves/seed/stem and it supplemented with sucrose for adjusting TSS to 5°Brix. It was then incubated overnight as shake culture (150 rpm) at 30±2˚C.

3.2.2.3. Fermentation of Jamun extracts

After processing and preparing the extracts from Jamun stem, leaves seed and pulp, the total soluble solids (TSS) of the respective extracts were adjusted to 20˚ Brix using cane sugar and pH was adjusted at 4.5 using citric acid/sodium bicarbonate. This was followed by the supplementation of potassium metabisulphite @ 100 ppm and 10 (% v/v) inoculum having 10^8 viable cells of *S. cerevisiae*. The viable cell count was noted after staining the cells with methylene blue (0.5%) and observing the cells in haemocytometer. The fermentations were carried out at 30±2˚C for 10 days in stationary state. 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, total sugar, ethanol as discussed below:

3.2.2.3.1. Estimation of total soluble solids

TSS was measured in terms of Brix, using ERMA hand refractometer having a range of 0-32°Brix.

3.2.2.3.2. 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 a reference to calibrate.

3.2.2.3.3. Estimation of total sugars

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

Reagents

Sodium potassium tartarate ($NaKC_4H_4O_6$) = 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 ($CuSO_4$) = 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)). 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 conc. 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 made 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.

3.2.2.3.4. Estimation of ethanol

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

Preparation of Potassium dichromate reagent

Potassium dichromate ($K_2Cr_2O_7$)	34.0 gm
Sulphuric acid (H_2SO_4)	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^oC. The distillate was collected in 50 mL volumetric flask containing 25 mL of $K_2Cr_2O_7$ reagent and it was kept at receiving end of the distillation unit. 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 $K_2Cr_2O_7$ separately. The distillate of samples and standards were heated in water bath at 70^oC 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. The standard curve was plotted considering the concentration against absorbance. (Appendix 1)

Fermentation efficiency was calculated as:

$$\text{Fermentation Efficiency (FE)} = \frac{\text{Ethanol content in wine}}{\text{Maximum ethanol obtainable from sugar utilized}} \times 100$$

Ethanol 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.

3.2.2.3.5. Determination of total phenolic compounds

The total phenolics content of the wine was determined by the Folin–Ciocalteau colorimetric method (Rathee *et al*., 2006). The Folin-Ciocalteu 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 2).

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 20% Na_2CO_3 was added then the mixture was allowed to stand for 30 min 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).

3.3. Standardization of various process parameters for the production of Jamun wines

Process standardisation of fermentation for Jamun wine variants was done by optimizing various environmental and cultural conditions known to affect yeast growth and the alcohol production. 100 mL the respective extract 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 1×10^8/mL and were subjected to batch fermentation for 10 days and incubated at 30±2°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 by one factor at a time (OFAT) approach. The optimum factors observed were used for the consecutive experiments.

1. The effect of temperature was studied by incubating the extracts based production media, after inoculation, at different temperatures including 15°C, 20°C, 25°C, 30°C, 35°C and 40°C.
2. The effect of pH was studied by varying the pH (3.0-6.0) of the production media using citric acid or sodium bicarbonate.
3. The effect of inoculum level was studied by inoculating the production media with varying levels of inoculums (2-14% v/v) having 10^8 viable cells/mL.
4. The effect of sugar concentration was studied by adjusting the solid content in the production media at 10, 15, 20 25 and 30°B with cane sugar.

3.3.2. Standardization of factors affecting Jamun wines, employing statistical tools

Plackett Burmann Design (PB) and Response Surface Methodology (RSM) are very useful and powerful statistical tools, widely employed for identifying the major independent variables or factors and their interactions among other factors that have a significant effect on a particular response (Plackett and Burman, 1946). In the present study, PBD was first employed for the primary screening and identification of significant parameters affecting the fermentation of Jamun wine variants.

3.3.2.1 Statistical optimization of stem wine production

The first PB was employed to the production of stem wine. This design was made by taking 19 process factors into account and each factor varied over 2 levels (high and low),

for the preliminary screening, to find out if there is little or no effect on ethanol content, total phenolic content (TPC) and total antioxidant capacity (TAC) due to any of the factors. The design consisted of 19 experimental runs and performed with 15% (w/v) of Jamun stem in 100mL deonised water (DW) with a pH of 4.5 taken as basal media with other media components added as shown in Table 3.1. The experiment was performed in one block and the variables were varied over two levels (high and low values) with values stated in Table 3.2. The design was based on a first order model as follows:

$$Y = \beta o + \sum \beta i Xi$$

Where: Y is the estimated response, *β*o is the model intercept, *β*i is the linear regression coefficient, and Xi is the independent variable (Levien *et al.*, 2005).

Table 3.1 Randomized PB experimental design for evaluating factors influencing ethanol, TPC and TAC during stem wine production

Run	TSS	Incubation Days	Inoculum size	NH_4SO_4	Urea	DAP	Peptone	M.E.	Y.E.	S.M.	Tryptone	K_2HPO_4	$CaCl_2$	NaCl	KCl	$ZnSO_4$	KH_2PO_4	ADP	$MgSO_4$
1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1
2	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1
3	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	-1	-1	-1
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
5	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	+1
6	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	-1	+1	-1
7	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	-1	-1	-1
8	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	+1	+1
9	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	-1	+1	+1
10	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	-1	+1
11	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	+1	-1
12	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	+1	-1	+1
13	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	+1
14	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	+1	+1	+1
15	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	-1	-1
16	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	+1	+1	-1
17	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	+1	-1
18	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	+1	-1	+1
19	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1
20	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1

M.E., YE and SM denote malt extract, yeast extract and soybean meal, respectively.

Table 3.2 Levels of independent variables used for optimization of stem wine production in PB design

Variables	Levels	
	Low (-1)	**High (+1)**
X_1:TSS	20° Brix	25° Brix
X_2: Incubation time	5 days	10 days
X_3: Inoculum size	5 (% v/v)	10 (% v/v)
X_4:$(NH_4)_2SO_4$	0	0.1 (% w/v)
X_5: Urea	0	0.1 (% w/v)
X_6: Diammonium Hydrogen-o-phosphate	0	0.1 (% w/v)
X_7: Peptone	0	0.1 (% w/v)
X_8: Malt extract	0	0.1 (% w/v)
X_9: Yeast extract	0	0.1 (% w/v)
X_{10}: Soyabean meal	0	0.1 (% w/v)
X_{11}: Tryptone	0	0.1 (% w/v)
X_{12}: K_2HPO_4	0	0.1 (% w/v)
X_{13}: $CaCl_2$	0	0.1 (% w/v)
X_{14}: NaCl	0	0.1 (% w/v)
X_{15}: KCl	5	0.1 (% w/v)
X_{16}: $ZnSO_4$	0	0.1 (% w/v)
X_{17}: KH_2PO_4	0	0.1 (% w/v)
X_{18}:Ammonium-diHydrogen-o-phosphate	0	0.1 (% w/v)
X_{19}: $MgSO_4$	0	0.1 (% w/v)

After screening the important process variables affecting ethanol level, TPC and TAC, the central composite design of RSM was created in order to develop a second order model using the parameters identified as significant by the PBD analysis. This design was consisted of 20 sets of experiments (6 centre points), with three highly significant variables: KH_2PO_4, yeast extract and $ZnSO_4$ based on the results obtained from PBD screening. The factors were examined at 5 levels as very high, high, centre, low and very low as shown in Table 3.3 with the values of the levels of each variable as stated in Table 3.4.

Table 3.3 CCD matrix of RSM for evaluating factors influencing ethanol, TPC and TAC of stem wine

Run	X_1 (KH_2PO_4)	X_2 (Yeast Extract)	X_3 ($ZnSO_4$)
1	0	2	0
2	-1	1	-1
3	0	0	0
4	0	0	0
5	-1	-1	-1
6	0	-2	0
7	1	-1	1
8	0	0	2
9	0	0	0
10	1	1	-1
11	-1	1	1
12	1	1	1
13	0	0	-2
14	2	0	0
15	1	-1	-1
16	0	0	0
17	0	0	0
18	-1	-1	1
19	0	0	0
20	-2	0	0

Table 3.4 Levels of independent variables used for optimization of stem wine production in RSM design

Variables	Levels (% w/v)				
	Very low	Low	Centre	High	Very high
	(-2)	(-1)	(0)	(+1)	(+2)
X_1: KH_2PO_4	0.05	0.75	0.1	0.125	0.15
X_2: Yeast Extract	0.05	0.75	0.1	0.125	0.15
X_3: $ZnSO_4$	0.05	0.75	0.1	0.125	0.15

The process variables were coded according to the following equation

$$x= Xi-X0/\Delta X$$

where x is the dimensionless coded value, Xi is the actual value of variables, X0 is the actual value of variables at the centre point and ΔX is the step change value. After the

conduct of the experiment, the data were fitted with a second-order polynomial equation as follow

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X^2_i + \sum \sum \beta_{ij} X_i X_j + \varepsilon$$

where Y is the predicted responses (ethanol, total phenolic content and total antioxidant capacity) β_0 is the model constant, β_i, β_{ii} and β_{ij} are model coefficients (linear, squared and interactive effects), ε and is the error. The resulting model was analysed using 'analysis of variance' (ANOVA), p and F values, PRESS values as well as the values of regression coefficients. 3D and Contour plots were also obtained by using Design Expert software version 8.0 to illustrate the relationship between the variables. Accuracy and general ability of polynomial model was evaluated by determination coefficient (R^2).

3.3.2.2. Statistical optimization of leaf wine production:

For optimization of leaf wine also, PBD was employed. This design was made by taking 16 process factors into account and each factor varied over 2 levels (high and low), for the preliminary screening, to find out if there is little or no effect on ethanol content, TPC and TAC due to any of the factors. The design also consisted of 20 experimental runs and was performed with 15% (w/v) of Jamun leaves in 100mL Deionised Water (DW) with a TSS of 25̊B and a pH of 4.5 taken as basal media in a 250 mL Erlenmeyer flask. The PB experimental design for optimization of nutritional factors in the production of leaf wine is given in Table 3.5. All the respective experiments were performed in one block and the variables were varied over two levels (high and low values) with values stated in Table 3.6. The designs were based on a first order model as follows:

$$Y = \beta o + \sum \beta i X i$$

Where, Y is the response, βo is the model intercept, βi is the linear coefficient, and Xi is the level of the independent variable (Levien *et al.*, 2005).

Table 3.5 Randomized PB experimental design for evaluating factors influencing ethanol, TPC and TAC during leaf wine production

Run	NH_4SO_4	Urea	DAP	Peptone	M.E.	Y.E.	S.M.	Tryptone	K_2HPO_4	$CaCl_2$	NaCl	KCl	$ZnSO_4$	KH_2PO_4	ADP	$MgSO_4$
1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1
2	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1
3	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	-1	-1	-1
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
5	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	+1
6	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	-1	+1	-1
7	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	-1	-1	-1
8	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	+1	+1
9	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	-1	+1	+1
10	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	-1	+1
11	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	+1	-1
12	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	+1	-1	+1
13	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	+1
14	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	+1	+1	+1
15	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	-1	-1
16	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	+1	+1	-1
17	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	+1	-1
18	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	+1	-1	+1
19	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1
20	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1

Table 3.6 Levels of independent variables used for optimization of leaf wine production in PB design

Variables	Levels	
	Low (-1)	High (+1)
X_1:$(NH_4)_2SO_4$	0	0.1 (% w/v)
X_2: Urea	0	0.1 (% w/v)
X_3: Diammonium Hydrogen-o-phosphate	0	0.1 (% w/v)
X_4: Peptone	0	0.1 (% w/v)
X_5: Malt extract	0	0.1 (% w/v)
X_6: Yeast extract	0	0.1 (% w/v)
X_7: Soyabean meal	0	0.1 (% w/v)
X_8: Tryptone	0	0.1 (% w/v)
X_9: K_2HPO_4	0	0.1 (% w/v)
X_{10}: $CaCl_2$	0	0.1 (% w/v)
X_{11}: NaCl	0	0.1 (% w/v)
X_{12}: KCl	0	0.1 (% w/v)
X_{13}: $ZnSO_4$	0	0.1 (% w/v)
X_{14}: KH_2PO_4	0	0.1 (% w/v)
X_{15}:Ammonium-diHydrogen-o-phosphate	0	0.1 (% w/v)
X_{16}: $MgSO_4$	0	0.1 (% w/v)

After screening the important process variables affecting ethanol level, TPC and TAC of leaf wine, the central composite designs of RSM were created in order to develop second order models using the parameters identified as significant by the PBD analysis. These designs were composed of 20 sets of experiments (6 centre points), with three highly significant variables. Table 3.7 shows a common design used for RSM employment on the production of leaf wine. The significant factors K_2HPO_4 (X_1), DAP (X_2) and malt extract (X_3) were chosen on the basis on PB and their concentration were further optimized, as discussed below.

Table 3.7 CCD matrix of RSM for evaluating factors influencing ethanol, TPC and TAC of leaf wine

Run	X_1 (K_2HPO_4)	X_2 (DAP)	X_3 (Malt extract)
1	0	2	0
2	-1	1	-1
3	0	0	0
4	0	0	0
5	-1	-1	-1
6	0	-2	0
7	1	-1	1
8	0	0	2
9	0	0	0
10	1	1	-1
11	-1	1	1
12	1	1	1
13	0	0	-2
14	2	0	0
15	1	-1	-1
16	0	0	0
17	0	0	0
18	-1	-1	1
19	0	0	0
20	-2	0	0

The factors were examined at 5 levels as very high, high, centre, low and very low as shown in Table 3.7 with the values of the levels of each variable as stated in Table 3.8.

Table 3.8 Levels of independent variables used for optimization of leaf wine production in RSM design

Variables	Levels (% w/v)				
	Very low	Low	Centre	High	Very high
	(-2)	(-1)	(0)	(+1)	(+2)
X_1	0.05	0.75	0.1	0.125	0.15
X_2	0.05	0.75	0.1	0.125	0.15
X_3	0.05	0.75	0.1	0.125	0.15

The process variables were coded according to the following equation

$$x = Xi - X0/\Delta X$$

where x is the dimensionless coded value, Xi is the actual value of variables, X0 is the actual value of variables at the centre point and ΔX is the step change value. After the conduct of the experiment, the data were fitted with a second-order polynomial equation as follow

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X^2_i + \sum\sum \beta_{ij} X_i X_j + \varepsilon$$

where Y is the predicted responses (ethanol, total phenolic content and total antioxidant capacity) β_0 is the model constant, β_i, β_{ii} and β_{ij} are model coefficients (linear, squared and interactive effects), ε and is the error. The resulting model was analysed using 'analysis of variance' (ANOVA), p and F values, PRESS values as well as the values of regression coefficients. 3D and Contour plots were also obtained by using Design Expert software version 8.0 to illustrate the relationship between the variables. Accuracy and general ability of polynomial model was evaluated by determination coefficient (R^2).

3.3.2.3. Statistical optimization of seed wine production:

For optimization of leaf wine also, PBD was employed. This design was made by taking 16 process factors into account and each factor varied over 2 levels (high and low), for the preliminary screening, to find out if there is little or no effect on ethanol content, TPC and TAC due to any of the factors. The design also consisted of 20 experimental runs and was performed with 15% (w/v) of Jamun seed in 100mL DW with a TSS of 25°B and a pH of 4.5 taken as basal media in a 250 mL Erlenmeyer flask. The PB experimental

design for optimization of nutritional factors in the production of seed wine is given in Table 3.9. All the respective experiments were performed in one block and the variables were varied over two levels (high and low values) with values stated in Table 3.10. The designs were based on a first order model as follows:

$$Y = \beta o + \sum \beta i Xi$$

Where, Y is the response, βo is the model intercept, βi is the linear coefficient, and Xi is the level of the independent variable (Levien *et al.*, 2005).

Table 3.9 Randomized PB experimental design for evaluating factors influencing ethanol, TPC and TAC during seed wine production

Run	NH_4SO_4	Urea	DAP	Peptone	M.E.	Y.E.	S.M.	Tryptone	K_2HPO_4	$CaCl_2$	NaCl	KCl	$ZnSO_4$	KH_2PO_4	ADP	$MgSO_4$
1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1
2	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1
3	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	-1	-1	-1
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
5	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	+1
6	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	-1	+1	-1
7	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	-1	-1	-1
8	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	+1	+1
9	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	-1	+1	+1
10	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	-1	+1
11	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	+1	-1
12	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	+1	-1	+1
13	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	+1
14	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	+1	+1	+1
15	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	-1	-1
16	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	+1	+1	-1
17	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	+1	-1
18	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	+1	-1	+1
19	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1
20	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1

Table 3.10 Levels of independent variables used for optimization of seed wine production in PB design

Variables	Levels	
	Low (-1)	High (+1)
X_1:$(NH_4)_2SO_4$	0	0.1 (% w/v)
X_2: Urea	0	0.1 (% w/v)
X_3: Diammonium Hydrogen-o-phosphate	0	0.1 (% w/v)
X_4: Peptone	0	0.1 (% w/v)
X_5: Malt extract	0	0.1 (% w/v)
X_6: Yeast extract	0	0.1 (% w/v)
X_7: Soyabean meal	0	0.1 (% w/v)
X_8: Tryptone	0	0.1 (% w/v)
X_9: K_2HPO_4	0	0.1 (% w/v)
X_{10}: $CaCl_2$	0	0.1 (% w/v)
X_{11}: NaCl	0	0.1 (% w/v)
X_{12}: KCl	0	0.1 (% w/v)
X_{13}: $ZnSO_4$	0	0.1 (% w/v)
X_{14}: KH_2PO_4	0	0.1 (% w/v)
X_{15}:Ammonium-diHydrogen-o-phosphate	0	0.1 (% w/v)
X_{16}: $MgSO_4$	0	0.1 (% w/v)

After screening the important process variables affecting ethanol level, TPC and TAC of seed wine, the central composite design of RSM were created in order to develop second order models using the parameters identified as significant by the PBD analysis. These design were composed of 20 sets of experiments (6 centre points), with three highly significant variables. Table 3.11 shows a common design used for RSM employment on the production of seed wine. The significant Factors (X_1) K_2HPO_4, (X_2) DAP and (X_3) $MgSO_4$ were chosen on the basis on PB and their concentration were further optimized as discussed below.

Table 3.11 CCD matrix of RSM for evaluating factors influencing ethanol, TPC and TAC of seed wine

Run	X_1 (K_2HPO_4)	X_2 (DAP)	X_3 ($MgSO_4$)
1	0	2	0
2	-1	1	-1
3	0	0	0
4	0	0	0
5	-1	-1	-1
6	0	-2	0
7	1	-1	1
8	0	0	2
9	0	0	0
10	1	1	-1
11	-1	1	1
12	1	1	1
13	0	0	-2
14	2	0	0
15	1	-1	-1
16	0	0	0
17	0	0	0
18	-1	-1	1
19	0	0	0
20	-2	0	0

The factors were examined at 5 levels as very high, high, centre, low and very low as shown in Table 3.11 with the values of the levels of each variable as stated in Table 3.12.

Table 3.12 Levels of independent variables used for optimization of seed wine production in RSM design

Variables	Levels (% w/v)				
	Very low	Low	Centre	High	Very high
	(-2)	(-1)	(0)	(+1)	(+2)
X_1	0.05	0.75	0.1	0.125	0.15
X_2	0.05	0.75	0.1	0.125	0.15
X_3	0.05	0.75	0.1	0.125	0.15

The process variables were coded according to the following equation

$$x= Xi-X0/\Delta X$$

where x is the dimensionless coded value, Xi is the actual value of variables, X0 is the actual value of variables at the centre point and ΔX is the step change value. After the conduct of the experiment, the data were fitted with a second-order polynomial equation as follow

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X^2_i + \sum \sum \beta_{ij} X_i X_j + \varepsilon$$

where Y is the predicted responses (ethanol, total phenolic content and total antioxidant capacity) β_0 is the model constant, β_i, β_{ii} and β_{ij} are model coefficients (linear, squared and interactive effects), ε and is the error. The resulting model was analysed using 'analysis of variance' (ANOVA), p and F values, PRESS values as well as the values of regression coefficients. 3D and Contour plots were also obtained by using Design Expert software version 8.0 to illustrate the relationship between the variables. Accuracy and general ability of polynomial model was evaluated by determination coefficient (R^2).

3.3.2.4. Statistical optimization of pulp wine production:

For optimization of leaf wine also, PBD was employed. This design was made by taking 16 process factors into account and each factor varied over 2 levels (high and low), for the preliminary screening, to find out if there is little or no effect on ethanol content, TPC and TAC due to any of the factors. The design also consisted of 20 experimental runs and was performed with 50 mL of Jamun pulp mixed in 50 mL (D.W.) with a TSS of 25̊B and a pH of 4.5 taken as basal media in a 250 mL Erlenmeyer flask. The PB experimental design for optimization of nutritional fators in the production of seed wine is given in Table 3.13. All the respective experiments were performed in one block and the variables were varied over two levels (high and low values) with values stated in Table 3.14. The designs were based on a first order model as follows:

$$Y = \beta o + \sum \beta i X i$$

Where, Y is the response, βo is the model intercept, βi is the linear coefficient, and Xi is the level of the independent variable (Levien *et al.*, 2005).

Table 3.13 Randomized PB experimental design for evaluating factors influencing ethanol, TPC and TAC during pulp wine production

Run	NH_4SO_4	Urea	DAP	Peptone	M.E.	Y.E.	S.M.	Tryptone	K_2HPO_4	$CaCl_2$	NaCl	KCl	$ZnSO_4$	KH_2PO_4	ADP	$MgSO_4$
1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1
2	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1
3	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	-1	-1	-1
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
5	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	+1
6	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	-1	+1	-1
7	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	-1	-1	-1
8	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	+1	+1
9	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	-1	+1	+1
10	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	-1	+1
11	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	+1	-1
12	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	+1	-1	+1
13	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	+1
14	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	+1	+1	+1
15	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	-1	-1
16	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	+1	+1	-1
17	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	+1	-1
18	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	+1	-1	+1
19	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1
20	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1

Table 3.14: Levels of independent variables used for pulp wine optimization in PB design

Variables	Levels	
	Low (-1)	High (+1)
X_1:$(NH_4)_2SO_4$	0	0.1 (% w/v)
X_2: Urea	0	0.1 (% w/v)
X_3: Diammonium Hydrogen-o-phosphate	0	0.1 (% w/v)
X_4: Peptone	0	0.1 (% w/v)
X_5: Malt extract	0	0.1 (% w/v)
X_6: Yeast extract	0	0.1 (% w/v)
X_7: Soyabean meal	0	0.1 (% w/v)
X_8: Tryptone	0	0.1 (% w/v)
X_9: K_2HPO_4	0	0.1 (% w/v)
X_{10}: $CaCl_2$	0	0.1 (% w/v)
X_{11}: NaCl	0	0.1 (% w/v)
X_{12}: KCl	0	0.1 (% w/v)
X_{13}: $ZnSO_4$	0	0.1 (% w/v)
X_{14}: KH_2PO_4	0	0.1 (% w/v)
X_{15}:Ammonium-diHydrogen-o-phosphate	0	0.1 (% w/v)
X_{16}: $MgSO_4$	0	0.1 (% w/v)

After screening the important process variables affecting ethanol level, TPC and TAC of pulp wine, the central composite design of RSM were created in order to develop second order models using the parameters identified as significant by the PBD analysis. These design were composed of 20 sets of experiments (6 centre points), with three highly significant variables. Table 3.15 shows a common design used for RSM employment on the production of pulp wine. The significant Factors (X_1) $MgSO_4$, (X_2) yeast extract and (X_3) KH_2PO_4 were chosen on the basis on PB and their concentration were further optimized as discussed below.

Table 3.15 CCD matrix of RSM for evaluating factors influencing ethanol, TPC and TAC of pulp wine

Run	X_1 ($MgSO_4$)	X_2 (Yeast extract)	X_3 (KH_2PO_4)
1	0	2	0
2	-1	1	-1
3	0	0	0
4	0	0	0
5	-1	-1	-1
6	0	-2	0
7	1	-1	1
8	0	0	2
9	0	0	0
10	1	1	-1
11	-1	1	1
12	1	1	1
13	0	0	-2
14	2	0	0
15	1	-1	-1
16	0	0	0
17	0	0	0
18	-1	-1	1
19	0	0	0
20	-2	0	0

The factors were examined at 5 levels as very high, high, centre, low and very low as shown in Table 3.15 with the values of the levels of each variable as stated in Table 3.16.

Table 3.16 Levels of independent variables used for optimization of pulp wine production in RSM design

Variables	Levels (% w/v)				
	Very low	Low	Centre	High	Very high
	(-2)	(-1)	(0)	(+1)	(+2)
X_1	0.05	0.75	0.1	0.125	0.15
X_2	0.05	0.75	0.1	0.125	0.15
X_3	0.05	0.75	0.1	0.125	0.15

The process variables were coded according to the following equation

$$x = Xi - X0/\Delta X$$

where x is the dimensionless coded value, Xi is the actual value of variables, X0 is the actual value of variables at the centre point and ΔX is the step change value. After the conduct of the experiment, the data were fitted with a second-order polynomial equation as follow

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X^2_i + \sum \sum \beta_{ij} X_i X_j + \varepsilon$$

where Y is the predicted responses (ethanol, total phenolic content and total antioxidant capacity) β_0 is the model constant, β_i, β_{ii} and β_{ij} are model coefficients (linear, squared and interactive effects), ε and is the error. The resulting model was analysed using 'analysis of variance' (ANOVA), p and F values, PRESS values as well as the values of regression coefficients. 3D and Contour plots were also obtained by using Design Expert software version 8.0 to illustrate the relationship between the variables. Accuracy and general ability of polynomial model was evaluated by determination coefficient (R^2).

3.4. Production of Jamun wines under optimized process conditions

Jamun wine variants were produced by selecting the optimized fermentation parameters and following the steps mentioned in section 3.2.1. In brief, the respective extracts of stem, leaves, seed and pulp were prepared as described in Section 3.2.1.1 and were supplemented with cane sugar to raise TSS to 20° Brix and pH adjusted to 4.5 using citric acid/sodium bicarbonate were subjected to batch fermentation. One litre of medium containing 20^oB TSS was taken in 2L Erlenmeyer flask, seeded with 10% (v/v) inoculum of *S. cerevisae,* supplemented with 100 ppm sodium metabisulphite. In case of stem wine, the basal media was supplemented with KH_2PO_4 (0.108 % w/v), yeast extract (0.05 % w/v) and $ZnSO_4$ (0.089 % w/v). Similarly, Jamun leaf wine was produced and stored, the only difference was that the nutritional factors supplemented were malt extract (0.063 % w/v), KH_2PO_4 (0.121 % w/v) and DAP (0.145 % w/v). In case of seed wine malt extract (0.063 % w/v), KH_2PO_4 (0.121 % w/v) and DAP (0.145 % w/v) were supplemented and in pulp wine yeast extract (0.075 % w/v), K_2HPO_4 (0.149 % w/v) and $MgSO_4$ (0.05 % w/v) were used. The media were then incubated in a stationary state after plugging with cotton wool in a BOD incubator at 25 ± 2^oC for 20 days. The contents of the bottles were mixed 2-3 times a day and the progress in fermentation were noted at regular intervals of 24h by analysing TSS, pH, sugar content, ethanol and total phenolic content as per the

methods described in section 3.2.2.3. After completion of fermentation, the wines were clarified, by repeated siphoning which was carried out 4 times with a sedimentation period of 7 days between each siphoning and stored in amber colored glass bottle for further use. The wine was analysed quantitatively for various constituents including total soluble proteins and total titratable acidity. Phytochemical screening Jamun wines was also performed qualitatively and were evaluated for the presence of tannins, terpenoids, flavonoids, saponins, glycosides, alkaloids and polysaccharides as per standard protocols.

3.4.1 Analytical methods

3.4.1.1. Estimation of titratable acidity

It was expressed as percent acidity and analyzed using method of Amerine *et al*., (1980). Titratable 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.

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

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

Solution A	:	2% Na_2CO_3 in 0.1 N NaOH.
Solution B	:	$CuSO_4$ (1%).
Solution C	:	Sodium potassium tartarate (2%).
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 3).

3.4.1.3. Total antioxidant capacity of wine

Total antioxidant capacity 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 $FeSO_4.7H_2O$ (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)

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 $FeSO_4.7H_2O$ (0.01-1µmol).

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 10mmol/L TPTZ (2,4,6- tripyridyl-S-triazine) in 40mmol/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.

3.4.1.4. Qualitative analysis of phytochemicals in Jamun wines

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

a) **Tannins:** To 0.5 mL of test solution, 1mL 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% NH_3 solution. Yellow colour formation was an indication for the presence of flavonoids (Krishnaiah *et al*., 2009).

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 ($HgCl_2$ - 1.36 gm, KI - 5 gm, Distilled water - 100 mL) were added. White yellowish precipitate was observed for the presence of alkaloids. (Sofowara, 1993).

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 Jamun wines

Ageing of the wines was done with or without oakwood chips (20chips/L with dimension 2×2×2 cm) in amber colored glass bottles for one year. The bottles were filled up to the brim and stored in dark. The wines were analyzed for bio-chemical parameters at regular intervals of one month for one year of ageing, by methods discussed in section 3.2.2.3 and 3.4.1.

3.5. Liquid Chromatography-Mass Spectrometry (LC-MS) profiling of Jamun wines

The MS spectrometry was carried out by LC-MS instrument (Waters Micromass Q-Tof Micro) having quadrapole time of flight, mass equipped with electrospray ionization. 20µL of the wine/extract sample was separated on a C18 column (250 mm × 4.6 mm 5 mm) using a mobile phase composed of methanol (A) – 0.1% formic acid (B) under gradient elution. The linear gradient increased from 10 to 17% A in 7 min, and increased to 30% A in another 1 min. Then, the solution A was continuously increased from 30 to 90% in 12 min. Finally, the solution A was linearly decreased back to the initial condition of 10%. Analysis was performed in total ion chromatography mode (TIC) with positive ESI interface. For MS, following parameters were used, Desolvation Gas: 550L/Hr, Cone Gas: 35 L/Hr, Desolvation Temperature: 300˚C, Source Temperature: 110˚C, Capillary Voltage: 3000V, Cone Voltage: 30V. Identifications were done on the basis of the molecular ion mass and comparison with literature data.

3.6. Assessment of safety of prepared Jamun wines

The safety of prepared Jamun wines was assessed by oral administration of the wines to the respective animal groups, each comprising of at least 7 rats and monitoring their renal and liver function tests as well as liver and kidney histology. Each rat of the groups was orally fed with two doses of 4mL/kg and 7 mL/kg bodyweight of the respective Jamun wines, once a day for 4 weeks.

3.6.1. Measurement of Hepatic and Renal functions

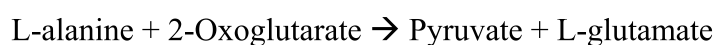
Blood was collected from the retro-orbital plexus of wine fed rats using fine heparinized glass capillary. For harvesting serum, blood samples were collected in vials and were kept in a BOD incubator at 37°C for 1h to allow clotting, after which they were centrifuged at 2000g for 20 min at 4°C. For plasma separation, blood samples were collected in heparinized vials (Beckett Denkiser) and were centrifuged at 1000g for 10 min at 4°C.

The essential biochemical indicators of kidney and liver function including aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, uric acid, alkaline phosphatase (ALP), creatinine, urea and uric acid were estimated using standard kits. The methods followed are described below.

3.6.1.1. Determination of alanine aminotransferase (ALT) activity (Reitman and Frankel, 1957)

<u>Principle</u>

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.



L-alanine + 2-Oxoglutarate → Pyruvate + L-glutamate

<u>Procedure</u>

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).

<u>Calculations</u>

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

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

Where:

T.V. = Total reaction volume

S.V. = Sample volume

Absorptivity = Millimolar Absorptivity of NADH at 340 nm =6.22

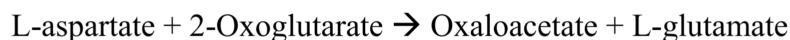
P = Cuvette light path (cm) = 1 cm

Activity of ALT (IU/L) = (ΔA/ min) × Factor (1768)

3.6.1.2. Determination of aspartate aminotransferase (AST) activity (Doumas and Biggs, 1969)

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.



L-aspartate + 2-Oxoglutarate → Oxaloacetate + 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.

Calculations

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

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

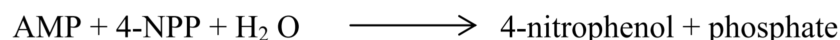
Where:

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)	=	(ΔA/ min) × Factor (1768)

3.6.1.3. Determination of alkaline phosphatase (ALP) activity (King and King, 1954)

Principle

This method utilises 4-nitrophenyl phosphate as the substrate. Under optimised conditions ALP present in the sample catalyses the following reaction



AMP + 4-NPP + H_2O ⟶ 4-nitrophenol + phosphate

At the pH of the reaction, 4-nitrophenol has an intense yellow colour. The reagent also contains a metal ion buffer system to ensure that optimal concentrations of Zinc and Magnesium are maintained. The metal ion buffer can also chelate other potentially inhibitory ions which may be present. The reaction is monitored by measuring the rate of

increase in absorbance at 405 nm which is proportional to the activity of ALP in the serum.

Procedure

To 1.0 mL of ALP reagent, 0.02 mL of serum sample was added, mixed and change in absorbance per min (ΔA/min) was read at 405 nm. Enzyme activity was expressed as IU/L.

Calculations

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

Activity of ALP (IU/L) = (ΔA/ min.) × Factor (2764)

3.6.1.4. Determination of creatinine (Jaffe, 1886)

Principle

Creatinine reacts with alkaline picrate to produce an orange-yellow color (Jaffe's reaction). The absorbance of the color formed is directly proportional to creatinine concentration and is measured at 500-520 nm.

Procedure

To 1.0 mL of working reagent, 0.01 mL of serum sample was added, mixed. Absorbance as noted at 505 nm after 20 seconds of mixing and again after 80 seconds. Enzyme activity was expressed as mg/dL.

Calculations

$$\text{Activity of creatinine (mg/dL)} = \frac{\Delta A\ \text{test}}{\Delta A\ \text{standard}} \times \text{concentration of standard (mg/dL)}$$

3.6.1.5. Determination of Urea (Berthelot, 1859)

Principle

Urease splits urea into ammonia and carbon dioxide. Ammonia released reacts with hypochlorite and phenolic chromogen to produce green color. The absorbance of this color is read at 578 nm.

Procedure

To 1.0 mL of enzyme solution, 0.01 mL of serum sample was added, mixed. It was incubated at 37°C for 30 min after which 1 mL of chromogen solution was added and

again incubated at 37°C for 5 min. Absorbance was noted at 578 nm. Urea concentration was expressed in mg%.

Calculations

$$\text{Urea (mg\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of sample}} \times 40$$

Blood urea nitrogen (BUN) was also calculated using the following formula

$$\text{BUN (mg\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of sample}} \times 18.69$$

3.6.1.6. Determination of uric acid

Principle

Uricase converts uric acid into allantoin and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidatively couples with phenolic chromogens to form a red colored compound, which has maximum absorbance at 510 nm. The concentration of red compound is directly proportional to that of the uric acid in the specimen. Uric acid was expressed in mg%

Procedure

To 1.0 mL of reagent, 0.025 mL of serum sample was added, mixed and incubated at 37°C for 5 min. Absorbance was noted at 510 nm. Urea concentration was expressed in mg%.

Calculations

$$\text{Uric acid (mg\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of sample}} \times 6$$

3.6.2. Microscopic examination of hepatic and renal histo-architecture

Livers and kidneys were removed aseptically from the animals and rinsed in isotonic saline solution. A part of the liver and a kidney section 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).

3.7. Organoleptic evaluation of prepared Jamun wines

The acceptability of the wine variants matured with and without oakwood chips were ascertained by a panel of 10 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 panellists were instructed to evaluate each sample by blind tasting as per score card.

Sensory evaluation

The wine samples were evaluated by a panel of 10 judges. The numerical scoring method and the score card followed are given below:

Score card

Code of the sample	:	
Appearance	:	2
Colour	:	2
Aroma	:	2
Bouquet	:	2

Acescent	:	2
Total acidity	:	2
Sugar	:	1
Body	:	1
Flavour	:	2
Astringency	:	2
General quality	:	2
Total score	:	20

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.

3.8. Evaluation of Jamun wines against diabetes mellitus in rat model

The anti-diabetic efficacy of Jamun wines was assessed in male Wistar rats.

3.8.1. Induction of diabetes

Streptozotocin (STZ) was used as a chemical agent for induction of diabetes. For standardization of STZ dose, a range of 40, 45, 50, 55 and 60 mg/kg bodyweight (bw) of STZ prepared in citrate buffer (pH 4.4, 0.1 M) were injected in the 5 rat groups comprising of 7 animals in each group. Before inducing diabetes, rats were fasted for at least 16 h. After 48 h of the injection, fasting blood glucose (FBG) levels were measured with enzymatic glucose oxidase-peroxidase (GOD-PAP) diagnostic kit (Freestyle optima, Abott India), after collecting blood samples through tail vein. The rats displaying plasma glucose levels higher than 250 mg/dl (Kuhad and Chopra, 2008) were considered as diabetic. The STZ dose displaying establishment of diabetes and non-mortality of rats for up to 30 days in more than 90% of the subjects was selected for conducing further experiments.

3.8.2. Experimental design

Rats were randomly distributed into 25 groups of seven rats each. Each group received the respective treatment once a day for 4 weeks at a daily single dose of 4ml/kg

bodyweight (equivalent to 280ml/70kg bodyweight, per day). All the treatments were given orally with the help of a gauge at 10:00 am daily except Insulin treatment which was given by intra-peritoneal route. Different treatment groups were as follows:

Control group - Each rat of this group received isotonic saline *per os* (p.o.) and citrate buffer (i.p.) at a daily dose of 4mL⁄kg.

Wine *per se* groups- Stem, leaf, seed and pulp wine were orally administered to healthy rats of the 4 respective animal groups, at a daily single dose of 4mL⁄kg, correspondingly.

Diabetic control group- Rats of this group were diabeticized and then were administered isotonic saline (p.o.) daily for a period of 4 weeks.

Wine prophylactic groups- There were 4 prophylactic groups, in which rats received the respective wine daily at a single dose of 4mL/kg for a period of 15 days followed by induction of diabetes and subsequent administration of wine again for 15 days.

Extract prophylactic groups- There were 4 prophylactic groups, in which rats received the respective extract daily at a single dose of 4mL/kg for a period of 15 days followed by induction of diabetes and subsequent administration of extrat again for 15 days.

Wine therapeutic groups- In these four animal groups, diabetes was first established and then the rats were fed with the respective wine at a daily single dose of 4mL⁄kg for a period of 4 weeks.

Extract therapeutic groups- In these four animal groups, diabetes was first established and then the rats were fed with respective extracts at a daily single dose of 4mL⁄kg for a period of 4 weeks.

Red wine treated group- Diabetes was first established and then the rats were fed with red wine respective extracts at a daily single dose of 4mL⁄kg for a period of 4 weeks.

Glibenclamide treated group- Diabetes was first established and then the rats were fed with a daily single dose of 0.5mg⁄kg of Glibenclamide for a period of 4 weeks (Lal *et al*., 2012).

Insulin treated group- Diabetes was first established and then the rats were injected with a daily single dose of 1IU/kg of Humulin, on alternate days for a period of 4 weeks (Yadav *et al*., 2014).

Fig. 3.1 Experimental design for evaluation of effect of Jamun wines on diabetes mellitus

3.8.3. Effect of wines on fasting blood glucose (FBG)

The efficacy of prepared wines was assessed on the FBG levels of non-diabetic and diabetic animals on weekly basis. For comparing the activity of wines with their extracts, red wine, Glibenclamide and Insulin, animals of these groups were also checked for their FBG levels on weekly basis. All animals were fasted for at least 16 h before the measurement. FBG was measured with enzymatic glucose oxidase-peroxidase (GOD-PAP) diagnostic kit (Freestyle optima, Abott India) method after collecting blood samples through tail vein.

3.8.3.1. Histological analysis of pancreas for assessing the effect of administration of Jamun wines on diabetic rats

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

3.8.4. Effect of wines on Insulin

Serum samples were analysed for measuring the concentration of insulin using a rat Insulin ELISA kit obtained from Crystal Chem (US).

3.8.5. Effect of wines on body weight (BW)

Induction of IDDM leads to loss of body weight. Therefore, BW of all the study subjects were also recorded on weekly basis.

3.8.6. Effect of Jamun wines on Lipid profile

Blood was collected from the retro-orbital plexus of the rats 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. Lipid profile analysis was done in serum to estimate the total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) using colorimetric kits obtained from Reckon India Pvt. Ltd.

3.8.6.1. Determination of Total Cholesterol (TC) (Taylor *et al*., 1978)

Principle

The cholesterol esters are hydrolysed to free cholesterol by cholesterol esterase (CE). The free cholesterol is then oxidised by cholesterol oxidase (CO) to cholesten 4-en-3-one with the simultaneous production of hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and phenolic compound in the presence of peroxidase to yield a coloured complex which is read at 505 nm.

Procedure

To 1 mL of Cholesterol reagent, 0.01 mL of sample/standard was added. It was mixed well and incubated at 37° C for 10 min and the absorbance was recorded at 505 nm.

Calculations

Cholesterol concentration was expressed in mg/dL and was calculated according to the following formula

$$\text{Cholesterol Concentration (mg/dl)} = \frac{\text{Absorbance of test x 200}}{\text{Absorbance of standard}}$$

3.8.6.2. Determination of Triglycerides (TG) (Fossati and Lorenzo, 1982)

Principle

Lipase hydrolyses triglycerides sequentially to di & monoglycerides and finally to glycerol. Glycerol Kinase (GK) using ATP as P04 source converts Glycerol liberated to Glycerol-3-Phosphate (G-3-Phosphate). G-3-Phosphate Oxidase (GPO) oxidises G-3-Phosphate & forms Dihydroxy acetone phosphate and hydrogen peroxide. Peroxidase (POD) uses the hydrogen peroxide formed, to oxidise 4- Aminoantipyrine and TOOS (N-ethyl-N-Sulphohydroxy propyl-m Toluidine) to a purple coloured complex. The absorbance of the coloured complex is measured at 520 nm (500-550 nm or with yellow filter) which is proportional to Triglyceride concentration.

Procedure

To 1 mL of working reagent, 0.02 mL of sample/standard was added and mixed. The reaction mixture was incubated at 37° C for 5 minutes and absorbance of test and standard against reagent blank was read at 520 nm. Triglycerides were expressed in mg/dL.

Calculations

$$\text{Triglycerides (mg/dL)} = \frac{\text{Absorbance of test x 200}}{\text{Absorbance of standard}}$$

3.8.6.3. Determination of HDL-C (Assmann *et al*., 1983)

Principle

HDL reagent is produced by using a combination of detergents and phosphorous compounds which specifically bind LDL, VLDL and chylomicron (CM) but not HDL. The combination protects LDL, VLDL and CM from the reaction by cholesterol esterase

and cholesterol oxidase. Consequently HDL-cholesterol is selectively exposed to react with both enzymes.

Procedure

0.2 mL of sample was taken in a micro-centrifuge tube and 0.2 mL of precipitation reagent was added to it. It was then centrifuged for 4000 rpm for ten minutes and the supernatant was separated. To 1 mL of working reagent, 0.05 mL of supernatant/standard was added. Contents were mixed and incubated at 37° C for 10 min. Absorbance was read at 505 nm and HDL-C was expressed in mg/dL.

Calculations

$$\text{HDL-C (mg/dl)} = \frac{\text{Absorbance of test} \times 50 \times 2}{\text{Absorbance of standard}}$$

Low-density lipoprotein-cholesterol (LDL-C) was calculated by the formula

$$\text{LDL-C} = \frac{TC}{1.19} + \frac{TG}{1.9} - \frac{\text{HDL-C}}{1.1} - 38$$ (Ahmadi *et al*., 2008)

Very low density lipoprotein cholesterol (VLDL-C) was calculated by using the formula

$$\text{VLDL-C} = \frac{TG}{5}$$

Atherogenic Index (AI) was calculated using the formula

$$\text{AI} = \log \frac{TG}{\text{HDL-C}}$$ (Nwagha et al., 2010)

3.8.7. Effect of Jamun wines on hepatic and renal Functions

Hepatic and renal function analysis were done by the estimating the levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline Phosphatase (ALP), creatinine, urea, blood urea nitrogen (BUN), and uric acid in the serum using commercial kits, as per the methods described in section 3.6.1.

3.8.7.1. Histological analysis of liver and kidney for assessment of the effect of Jamun wines on diabetic rats

Liver tissues and kidney were 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.

3.9. Effect of Jamun wines on oxidative stress

All the wines were evaluated for their anti-oxidative potential by determining the following parameters in the liver, kidney and brain homogenates of the diabetes model:

- Extent of lipid peroxidation (Wills, 1966)
- Activity of superoxide dismutase (SOD) (Kono, 1978)
- Levels of catalase (Luck *et al*., 1974)
- Levels of nitrite (Green *et al.,* 1982)

3.9.1. Preparation of tissue homogenate and post mitochondrial supernatant (PMS)

Livers, kidneys and brains were 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 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 homogenates were centrifuged at 10000 rpm for 20 min at 4°C. The supernatants thus obtained were called post mitochondrial supernatants (PMS).

3.9.2 Effect on lipid peroxidation

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-HCI buffer	:	0.1M Tris-HCI, 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 × 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 ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$).

3.9.3. 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_2CO_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.

3.9.4. Determination of catalase

The activity of catalse was measured according to the method given by Luck *et al*., 1974. For this 1.95mL of phosphate buffer (0.05M, pH 7) was taken with 1mL of H_2O_2 in a test tube and 5µL of PMS was added to it. Change in absorbance was recorded at 240nm and the results were expressed as mM of H2O2 decomposed/min/mg protein, using molar extinction coefficient of chromophore (0.0394 mM^{-1} cm^{-1}).

3.9.5. Determination of nitrite levels

The levels of nitrite were determined by the method given by Green *et al*., 1982. It was done by taking 400µL of the serum sample and adding 100µL DW to it and 500µL of Griess reagent (0.1(% w/v) Naphthylethylene diamine dihydrocholric acid and 1 (% w/v) sulphanilamide in 5 (% v/v) phosphoric acid in equal amounts). The reaction mixture was incubated at 37°C for 10 min and absorbance was recorded at 546 nm. Nitrite levels were expressed as µM of nitrite/mL of the sample.

3.10. Statistical analysis

All data is expressed as the mean ± SD of seven animals per group. Statistical differences between the groups were analyzed with SPSS software, version 11.0. Two-way analysis of variance (ANOVA) was performed for Blood Glucose levels and one-way analysis of variance (ANOVA) for lipid profile, hepatic and renal functions, followed by multiple comparisons using Holm-Sidak method. The level of significance was set at $p<0.05$.