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Research Papers

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Optimization of Process Parameters for Bioactive Metabolite Production by *Nocardiopsis trehalosi* VSM-13 Using Response Surface Methodology and Unstructured Kinetic Modelling

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Abstract

Nocardiopsis trehalosi VSM 13, an actinobacterium isolated from marine environment was tested for the optimum culture conditions in shake-flask fermentations using one-factor-at-a-time method. Response Surface Methodology (RSM) based Central composite Design was used to design the experiments, build the model and determine the optimum conditions for the desirable responses. RSM using a full factorial Box-Behnken design evaluated the optimized process conditions as 10 days of incubation time, pH - 8.0, temperature - 35^{0}C, fructose @ 1.5% (w/v) and yeast extract @ 1% (w/v) which influenced the bioactive metabolite production by *N. trehalosi* VSM 13 and RSM model obtained results (R^2 > 0.99) revealed a satisfactory correlation between the experimental and predicted values. Unstructured kinetic model-based parameters were also evaluated using non-linear regression method to test the fitness of the selected models. Parameters of growth and substrate utilization rates have shown excellent significant R^2 values of 0.996 and 0.994, respectively.

Keywords: *Nocardiopsis trehalosi*, Bioactive metabolites, Regression analysis, Statistical optimization.

Introduction

The potential sources for the commercially important bioactive compounds are the marine actinomycetes. They attracted great attention since they have developed unique metabolic and physiological capabilities that not only ensure survival in extreme habitats, but also offer the potential to produce compounds with antibacterial, antifungal, antimitotic, antiviral, cytotoxic and antineoplastic activities. Several therapeutic agents have been isolated from marine actinomycetes which are entirely new and unique when compared to their terrestrial counterparts (1, 2).

Screening of phylogenetically distinct and unique organisms from extreme ecosystems is a rational approach to discover novel chemical structures with medicinally relevant biological activities (3). In the recent literature the marine environment has emerged as a new frontier in natural products chemistry when there is an urgent need for novel drug templates to combat the emerging problem of drug resistance and cancer (4). Although the exploitation of marine actinomycetes as a source of novel secondary metabolites is in its infancy, the discovery rate of novel secondary metabolites from marine

actinomycetes has recently surpassed that of their terrestrial counterparts, as evident by the isolation of unique compounds like salinosporamide and abyssomycin from marine actinomycete genera *Salinispora* and *Verrucosispora* (5). In this regard there is a need to explore the unexplored marine ecosystems that are a wealthy source of novel actinomycetes capable of producing new important bioactive compounds.

Microorganisms vary in the ways they respond to fermentation conditions and once the requirements are suitable they may produce a wide variety of bioactive compounds. The necessary conditions for fruitful production of antimicrobial substances were distinctive microorganism, accurate fermentation conditions and sensitive methods for identification of activity (6). Evaluation on the biosynthesis of antimicrobial agents involves the optimization of culture media for their production that can be accomplished by a meticulous study of the suitability of physiological parameters and supplementary nutrition. Medium constituents and cultural parameters are the important factors for bioactive metabolite production (7). Variations in the type and nature of carbon, nitrogen or phosphate sources and trace elements have been reported to influence bioactive metabolite production (8). It is necessary to optimize nutrient and environmental conditions to achieve maximum production of antimicrobial agents by any producer strain (9, 10).

Classical and conventional methods used for optimization of the variables do not give the interactive effects of all the variables involved (11). These methods are time consuming and need application of a number of experiments to determine the optimized conditions which are inaccurate. In recent years a multivariate statistical and mathematical techniques have been adopted to identify the optimal combinations of factors and interactive effects between the variables (12). Response Surface Methodology (RSM), is the most commonly applied statistical and mathematical programme to determine the effect of the independent variables against the responses (12). A mathematical model is set of hypotheses which are natural phenomena that are translated into mathematical expressions (13).

Unstructured Kinetic mathematical models provide the complete knowledge of dynamic behaviour optimization and control the most fermentation processes. The designed unstructured models mimic the profiles of bioactive metabolite production and also explain the kinetic relationships between substrate, product and biomass. Batch-mode operated reactions are hard to model, owing to time-varying characteristics of cell systems, which often result in nonlinearities. Only total cell concentrations are considered in unstructured models and provide accurate description of whole fermentation. Parameters estimation is an essential step for model verification and subsequent use of mathematical models are successfully applied to study a variety of bioproduct productions (14-16). The paper explains the application of RSM by CCD to optimize the physical and chemical parameters and interactive effects of the variables and to evaluate the kinetic parameters for bioactive metabolite production by *Nocardiopsis trehalosi* VSM 13.To the best of our knowledge, no research publication is available on the production of bioactive compounds by *Nocardiopsis trehalosi* VSM 13 and its effect against the responses using RSM (*Staphylococcus aureus*, *Streptococcus mutans, Xanthomonas campestris, Pseudomonas aeruginosa and Candida albicans*) using the RSM.

Materials and Methods

***Isolation and identification of the strain*:** During the course of screening for bioactive metabolites from actinomycetes of marine habitats, the strain VSM-13 with distinct morphological features was isolated using Actinomycete isolation agar (AIA) medium (0.2% sodium caseinate, 0.05% glycerol, 0.01% L-asparagine, 0.4% sodium propionate, 0.05% disodium phosphate, 0.01% magnesium sulphate, 0.0001% ferrous sulphate and 2% agar) from deep sea sediment samples, collected from Bay of Bengal, north coastal Andhra Pradesh, India. The pure culture was maintained on AIA

slants (3% NaCl [w/v], pH 8) at 4° C. The strain was identified as *Nocardiopsis trehalosi* by polyphasic taxonomy. The rRNA sequence was deposited in the NCBI GenBank with an accession number KT153051.

***Statistical optimization design*:** The production of bioactive compounds by VSM 13 was optimized and developed applying CCD of RSM using Design Expert software version 8 (Stat-Ease Inc., Minneapolis, USA) to study the production of bioactive compounds by VSM 13 using process variables. The experimental design involves estimation of the coefficients in a mathematical model, predicting the response, and checking the adequacy of the model (17). Five independent variables such as incubation time, A; pH, B; Temperature, C; Concentration of fructose, D; and Concentration of yeast extract E were selected based on the experiments conducted with one-factor-at-a-time method (OFAT). Table 1 shows the levels and the ranges of the five independent variables coded to two levels namely: Low (-1) and High (+1).

The number of experiments *n* for *k* factors is represented as $n=k^3$. Experiment runs were randomized to reduce the effects of unexpected variability in the observed responses. The variables were coded as per the following equation:

$$X = \frac{(X_\alpha - X_0)}{\Delta X} \qquad (1)$$

Where X is the coded value for the variables, X_α is the corresponding actual value, X_o is the actual value in the centre of the domain and ΔX is the increment of X_α corresponding to a variation of 1 unit of X.

A total of 50 experiments were calculated using following equation that has 2^5 full factorial CCD for five variables comprising 32 factorial points, 10 axial points and 8 replicates.

$N = 2^n + 2n + n_c = 2^5 + 2 \text{ X } 5 + 8 = 50 \qquad (2)$

Where N is total number of experimental runs to be performed, n is number of variables (factors) and n_c is number of replicates at centre points.

After the experiments the data was fitted in to the second-order polynomial model presented in Eq. (3) which is a nonlinear computer generated quadratic model (18).

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \left(\sum_{i=1}^{n} \beta_{ii} X_i\right)^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \qquad (3)$$

Where *Y is* predicted response, β_0 *is intercept* coefficient, β_i is the linear coefficient, β_{ij} is the interaction coefficients, β_{ii} is the quadratic coefficients, and *Xi and Xj* are coded values of the five additive variables under study (19).

Analysis of Variance (ANOVA)

The model adequacy was analysed using the ANOVA (20) to determine the variable significance. The significance of the model was appraised by Fisher's satirical test (*F-test*). (21). Model adequacy of the responses was verified by evaluating the coefficient of determination (R^2), adjusted coefficient of determination (adj. R^2) and coefficient of variation (CV). The model and the regression coefficient were considered significant if the p-value < 0.05. The data with significance of more than 95% and *p*-value <0.05 was re fitted to obtain the final reduced model. Conformity of the experiments used to ratify the equations using the combination of different optimized parameters (21, 22).

By solving the regression equation and by evaluating the response surface and contour plots the optimum values of selected variables were obtained. The response surface and the contour plots obtained from the model are used for analysis of different interactive effects between the independent variables while keeping the value of the third variable constant to give accurate geometrical representation (23).

Model Development: Growth rate and utilization of the substrate limiting concentration influence the production of bioactive metabolite by *Nocardiopsis trehalosi* VSM 13. Models of Logistic and Luedeking-Piret were applied to simulate the cell growth and bioactive metabolite production of

the strain can be calculated from kinetic expressions. The unstructured model equation for *N. trehalosi* VSM 13 growth, optimized substrate utilization, bioactive metabolite production was obtained from data of (16). Experimental data obtained from batch shake-flask fermentations were used to estimate the kinetic parameters of bioactive metabolite production.

Results

Influence of different variables such as Incubation time, pH, Temperature, Concentration of Fructose and Yeast Extract on bioactive metabolite production and their effect against the five responses (*Staphylococcus aureus*, *Streptococcus mutans, Xanthomonas campestris, Pseudomonas aeruginosa and Candida albicans*) represented as zone of inhibition (mm) were scrutinized in detail and optimized.

***Optimization for the production of bioactive metabolite by VSM 13 using RSM*:** CCD of RSM was employed to study the interactive effects of the significant variables to determine the optimum values for the production of bioactive compound by VSM 13. Table 2 illustrates the experimental conditions of the variables and the corresponding responses along with both the predicted and the experimental values against the five responses. The results at each point are based on the experimental central composite (ECC) design and their corresponding predicted values that are presented in Table-2.

50 experiments were carried by randomized experimental run order to determines the effect of the five variables against characteristic five responses. The optimized conditions of the independent variables for the bioactive metabolite production by VSM 13 and its effect against the five responses (Inhibition of growth of the pathogenic micro-organisms by the bioactive compound produced by VSM 13 represented in mm) was experimentally found to be 24 mm for *S.aureus*, 22 mm for *S. mutans*, 21mm for *X. campestris*, 24 mm for *P. aeruginosa* and 25 mm for *C. albicans.* The optimized conditions of the independent variables was Time of incubation -10 days, pH - 8, temperature - 35^0C, fructose concentration - 1.5% and yeast extract concentration - 1%. The second degree polynomial models developed for the production of bioactive compounds by VSM 13 were represented in equations (4) - (8) to validate the coefficient of the model. The zones of inhibition by the bioactive metabolite produced by the strain were empirical functions of variables evaluated.

Response 1 (*Staphylococcus aureus*)

$$Y1 = -164.91705 + 8.56377A + 14.24954B + 3.93613C + 17.51620D + 21.21668E + 0.031250AB + 6.25000E\text{-}003AC - 0.062500AD - 0.062500\ AE + 6.25000E\text{-}003BC - 0.062500BD - 0.062500BE - 0.012500CD - 0.062500CE + 0.12500DE - 0.43793A^2 - 0.93793B^2 - 0.057517C^2 - 5.75172D^2 - 9.75172E^2 \quad (4)$$

Response 2 (*Streptococcus mutans*)

$$Y2 = -165.53202 + 14.20690A + 11.45375B + 3.31176C + 13.78149D + 13.07982E + 0.000000AB + 0.000000AC + 0.000000AD + 0.000000AE - 0.012500BC - 0.12500BD + 0.12500BE + 0.025000CD + 0.075000CE - 0.25000DE - 0.71034A^2 - 0.71034B^2 - 0.048414C^2 - 4.84138D^2 - 8.84138E^2 \quad (5)$$

Response 3 (*Xanthomonas campestris*)

$$Y3 = -108.40129 + 3.65213A + 10.67023B + 3.17703C + 11.04863D + 16.30203E - 0.062500AB + 0.000000AC + 0.000000AD + 0.000000AE + 0.000000BC + 0.000000BD + 0.000000BE + 0.025000CD - 0.025000CE + 0.25000DE - 0.15172A^2 - 0.65172B^2 - 0.046069C^2 - 4.60690D^2 - 8.60690E^2 \quad (6)$$

Response 4 (*Pseudomonas aeruginosa*)

$$Y4 = -203.76582 + 6.06897A + 21.36253B + 5.22318C + 20.64138D + 16.88347E + 0.000000AB + 0.000000AC + 0.000000AD + 0.000000AE - 0.025000BC + 0.000000BD + 0.25000BE + 0.000000CD - 0.050000CE + 0.000000DE - 0.30345A^2 - 1.30345B^2 - 0.072138C^2 - 7.21379D^2 - 9.21379E^2 \quad (7)$$

Response 5 (*Candida albicans*)

$Y5$= - 278.99919 + 15.70385A + 28.35132B + 4.95854C + 19.58702D + 25.23448E + 0.000000AB + 0.000000AC + 0.000000AD + 0.000000AE + 0.000000BC + 0.000000BD + 0.000000BE + 0.000000CD + 0.000000CE + 0.000000DE - 0.77931A^2 - 1.77931B^2 - 0.071172C^2 - 7.11724D^2 -13.11724E^2 (8)

*Y*1, *Y*2, *Y*3, *Y*4 and *Y*5 are responses (*Staphylococcus aureus*, *Streptococcus mutans, Xanthomonas campestris, Pseudomonas aeruginosa and Candida albicans*) whose growth is inhibited by the bioactive metabolite produced by VSM 13.

ANOVA

The significance of the fit of the quadratic model was analysed by ANOVA variance. ANOVA analysis showed that the confidence level of the model was greater than 95% while the *p*- value of the model was less than < 0.0001. The model with the *p*- value less than 0.05 was considered statistically significant and acceptable. The p-value of the model was found to be < 0.0001 for all the five responses was given Table 3 implying that the model is significant and the lack of fit test of the model is found to be insignificant which shows that the model is significant.

Regression analysis of the model could be measured by the coefficient of determination (R^2). The statistical regression analysis of the equation shows that the R^2 for all the five responses is give in table 4 and was found > 0.9 which indicate the model to be significant. Coefficient of Variation (CV) indicates the significance of the model. In the present investigation the CV of all the five responses is shown in Table 4. Adequate precision ratios of the models for bioactive metabolite production by VSM 13 and second-order quadratic model equations and regression coefficients (%) of all responses were given in Table 4. Variance analysis reveals the significant factors affecting the growth inhibition of the pathogenic micro-organisms (five responses) by the bioactive compounds produced by VSM 13 include incubation time (days), pH, temperature, concentration of fructose and concentration of yeast extract.

***3D plots to study the interaction effect on responses*:** 3D plots are generated to study the interactive effects among the five variables and to determine the optimum level of each variable for maximum response. Response surface 3D plots show the interactions of the two independent variables where the other variable is fixed at zero. Figs. 1-5 represent the response surface 3D plots generated for the production of bioactive compounds by VSM 13 and their effect against the five responses. The values obtained from the 3D plots were found to be almost similar with optimum values. The obtained optimum conditions were verified by running the experiments for validation (Table-2).

***Distribution of Residuals*:** Model adequacy was studied by the distribution of the residuals by determining whether the residuals followed a normal distribution. Initially the residuals were normalized with respect to their standard deviations (studentized). A normal distribution function was then fit to the studentized residuals. Then, the studentized residual predicted by the best-fit normal distribution was plotted against the experimentally obtained studentized residual in Fig. 6. Straight line formation indicates that the normal distribution of the residuals.

***Evaluation of fermentation constants using kinetic modelling*:** Non-linear regression using least-square method was applied to analyse the data obtained from experiments and Microsoft Excel solver 2010 was used to fit the data. Fig.7 (a) - (e) shows very good fit of the compared experimental versus model predicted zones of inhibition of bioactive metabolite analysed by agar disc diffusion methods against test pathogens such as *S. aureus*, *S. mutans, X. campestris, P. aeruginosa*, and *C. albicans. N. trehalosi* VSM 13 biomass growth, limiting substrate utilization results obtained from shake flask experiments and model kinetics were compared in Fig. 7 (f). A plot of Biomass (*N. trehalosi* VSM 13) function,

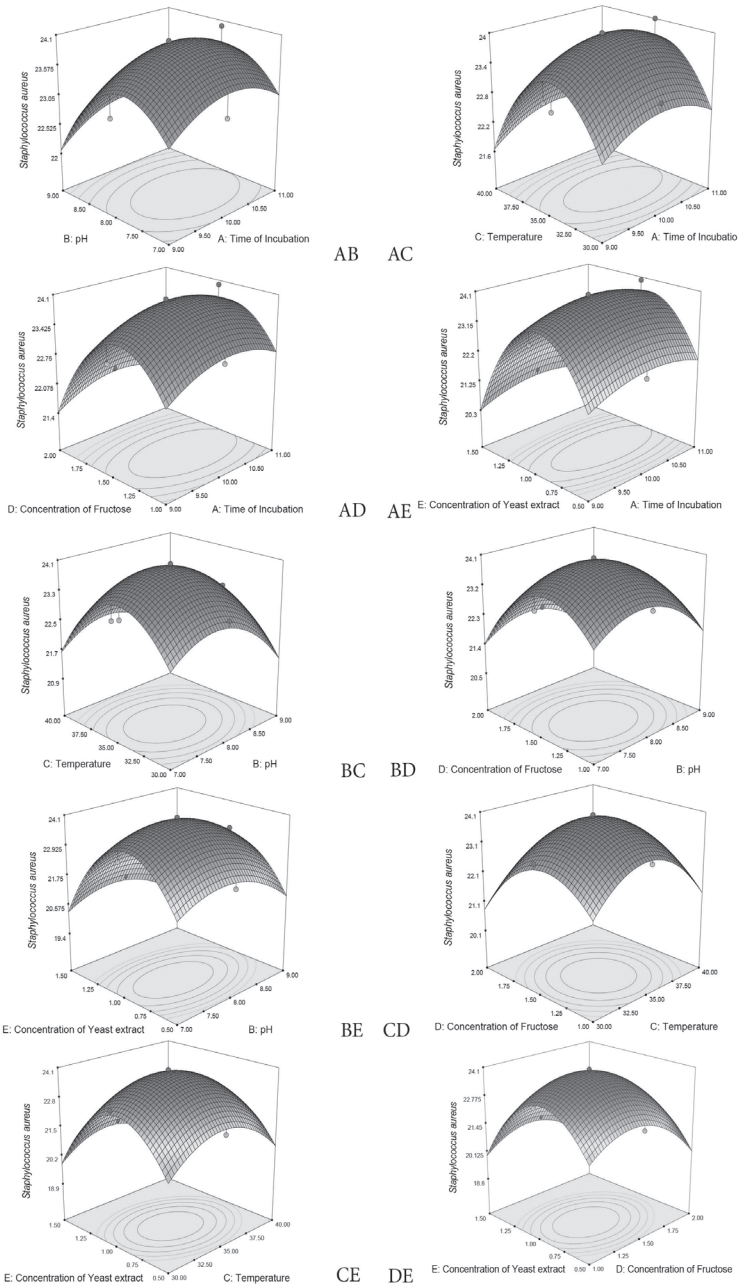

Fig. 1. Response surface plots consisting three-dimensional views and contours showing interactive effects of selective variables on Zone of Inhibition (mm) of the bioactive compound production by *Nocardiopsis trehalosi* VSM 13 against *Staphylococcus aureus:* (AB) Time of incubation and pH, (AC) Time of incubation and Temperature, (AD) Time of incubation and Concentration of fructose, (AE) Time of incubation and Concentration of Yeast extract, (BC) pH and Temperature, (BD) pH and Concentration of fructose, (BE) pH and Concentration of yeast extract, (CD) Temperature and Concentration of fructose, (CE) Temperature and Concentration of Yeast extract, (DE) Concentration of fructose and Concentration of Yeast extract.

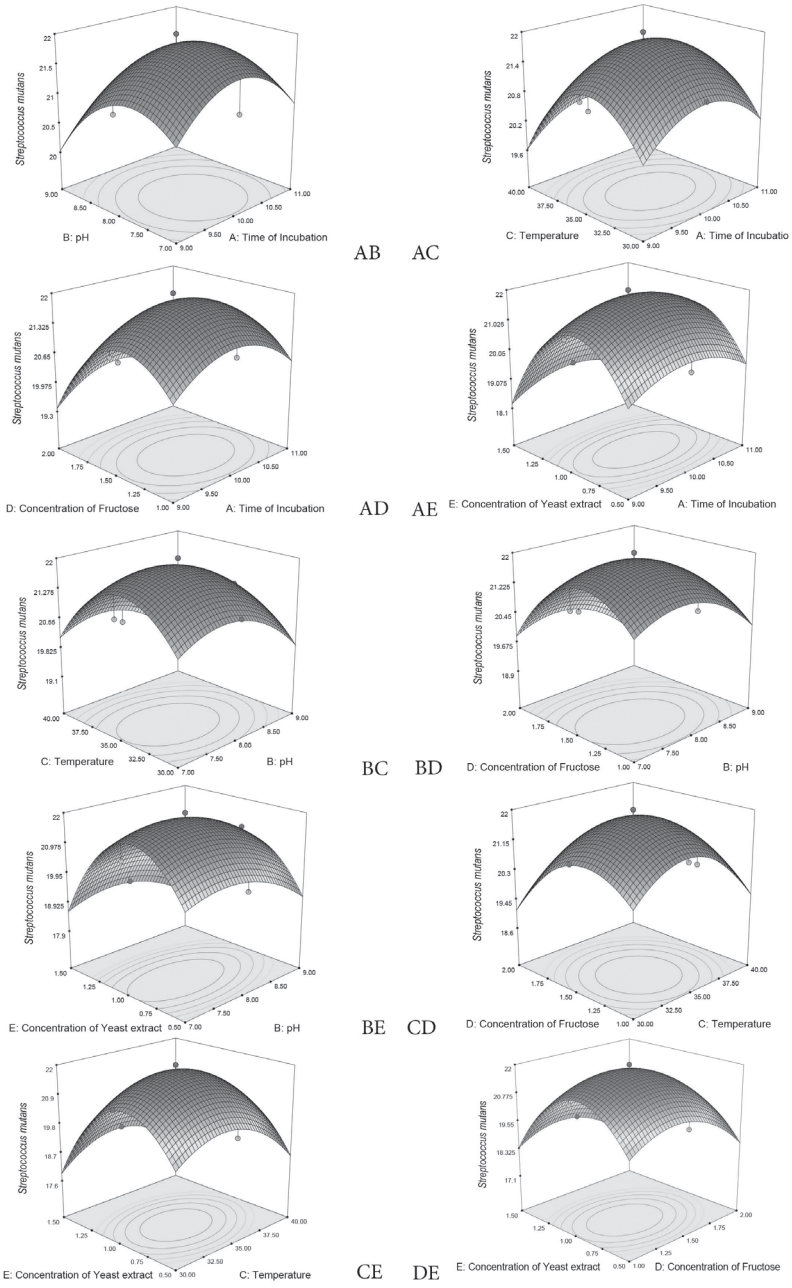

Fig. 2 Response surface plots consisting three-dimensional views and contours showing interactive effects of selective variables on Zone of Inhibition (mm) of the bioactive compound production by *Nocardiopsis trehalosi* VSM 13 against *Streptococcus mutans*: (AB) Time of incubation and pH, (AC) Time of incubation and Temperature, (AD) Time of incubation and Concentration of fructose, (AE) Time of incubation and Concentration of Yeast extract, (BC) pH and Temperature, (BD) pH and Concentration of fructose, (BE) pH and Concentration of yeast extract, (CD) Temperature and Concentration of fructose, (CE) Temperature and Concentration of Yeast extract, (DE) Concentration of fructose and Concentration of Yeast extract.

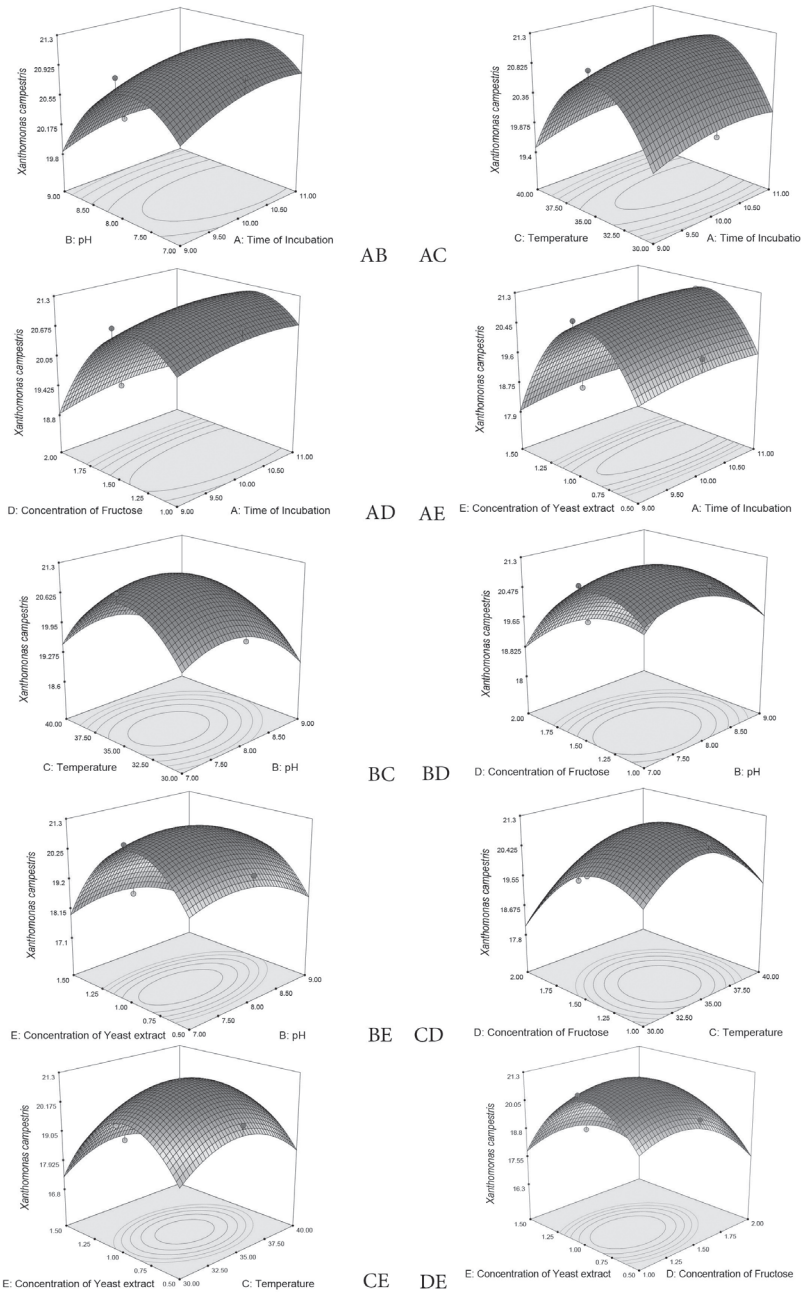

Fig. 3. Response surface plots consisting three-dimensional views and contours showing interactive effects of selective variables on Zone of Inhibition (mm) of the bioactive compound production by *Nocardiopsis trehalosi* VSM 13 against *Xanthomonas campestris*: (AB) Time of incubation and pH, (AC) Time of incubation and Temperature, (AD) Time of incubation and Concentration of fructose, (AE) Time of incubation and Concentration of Yeast extract, (BC) pH and Temperature, (BD) pH and Concentration of fructose, (BE) pH and Concentration of yeast extract, (CD) Temperature and Concentration of fructose, (CE) Temperature and Concentration of Yeast extract, (DE) Concentration of fructose and Concentration of Yeast extract.

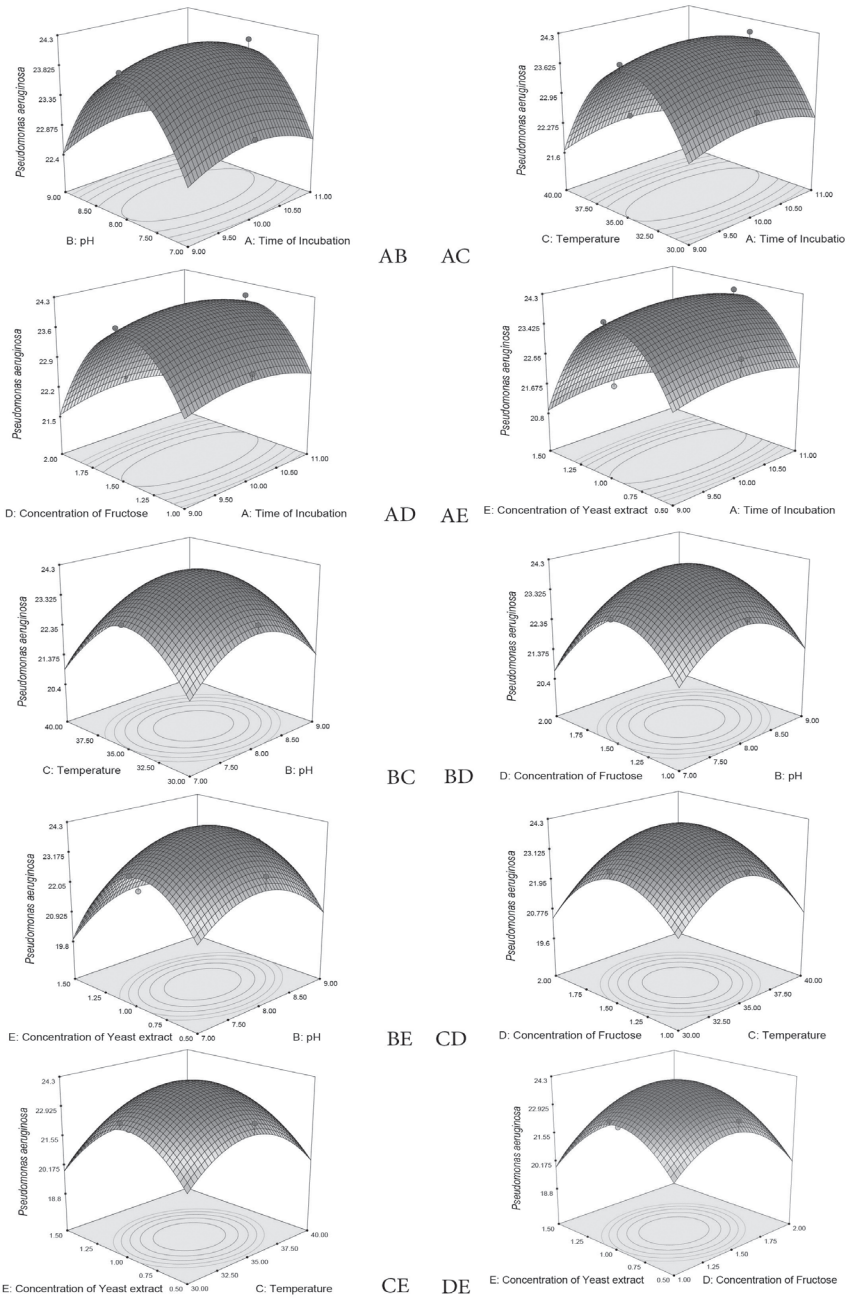

Fig. 4. Response surface plots consisting three-dimensional views and contours showing interactive effects of selective variables on Zone of Inhibition (mm) of the bioactive compound production by *Nocardiopsis trehalosi* VSM 13 against *Pseudomonas aeruginosa*: (AB) Time of incubation and pH, (AC) Time of incubation and Temperature, (AD) Time of incubation and Concentration of fructose, (AE) Time of incubation and Concentration of Yeast extract, (BC) pH and Temperature, (BD) pH and Concentration of fructose, (BE) pH and Concentration of yeast extract, (CD) Temperature and Concentration of fructose, (CE) Temperature and Concentration of Yeast extract, (DE) Concentration of fructose and Concentration of Yeast extract.

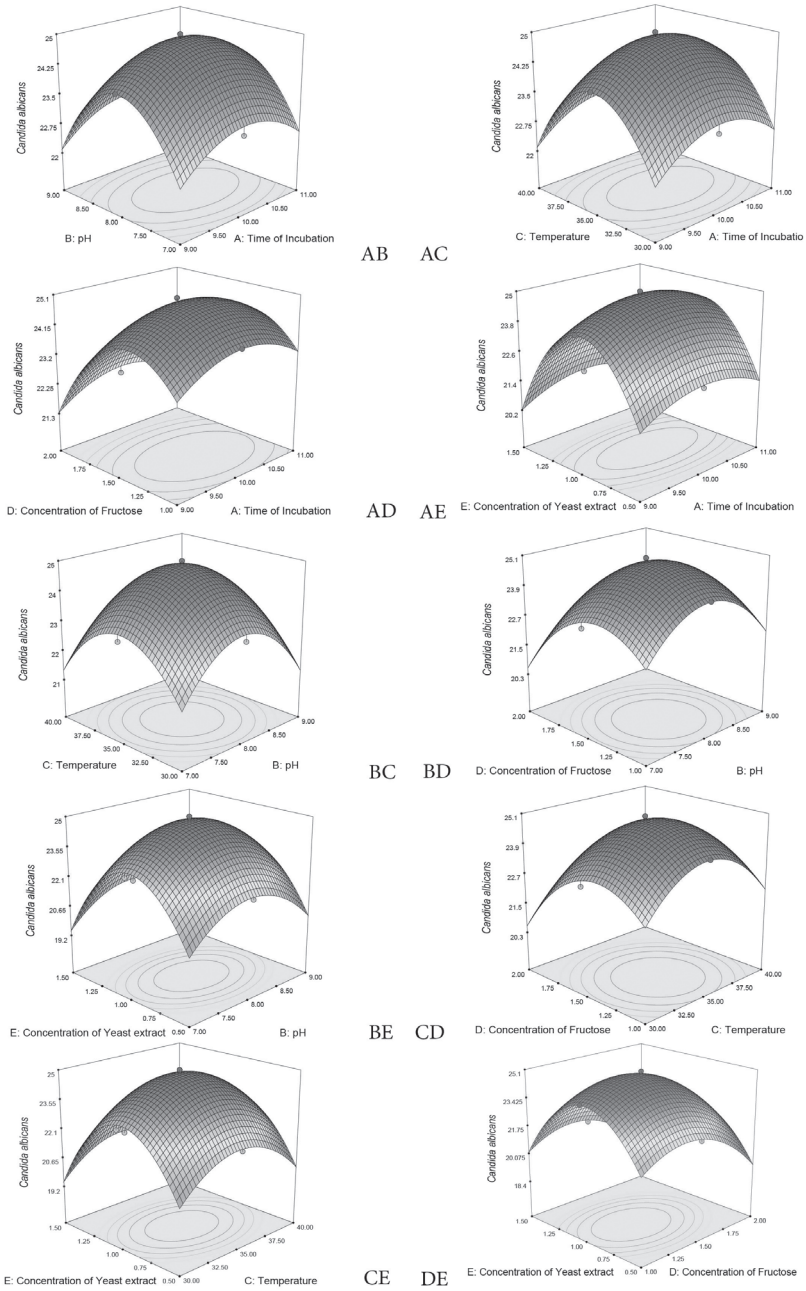

Fig. 5. Response surface plots consisting three-dimensional views and contours showing interactive effects of selective variables on Zone of Inhibition (mm) of the bioactive compound production by *Nocardiopsis trehalosi* VSM 13 against *Candida albicans*: (AB) Time of incubation and pH, (AC) Time of incubation and Temperature, (AD) Time of incubation and Concentration of fructose, (AE) Time of incubation and Concentration of Yeast extract, (BC) pH and Temperature, (BD) pH and Concentration of fructose, (BE) pH and Concentration of yeast extract, (CD) Temperature and Concentration of fructose, (CE) Temperature and Concentration of Yeast extract, (DE) Concentration of fructose and Concentration of Yeast extract.

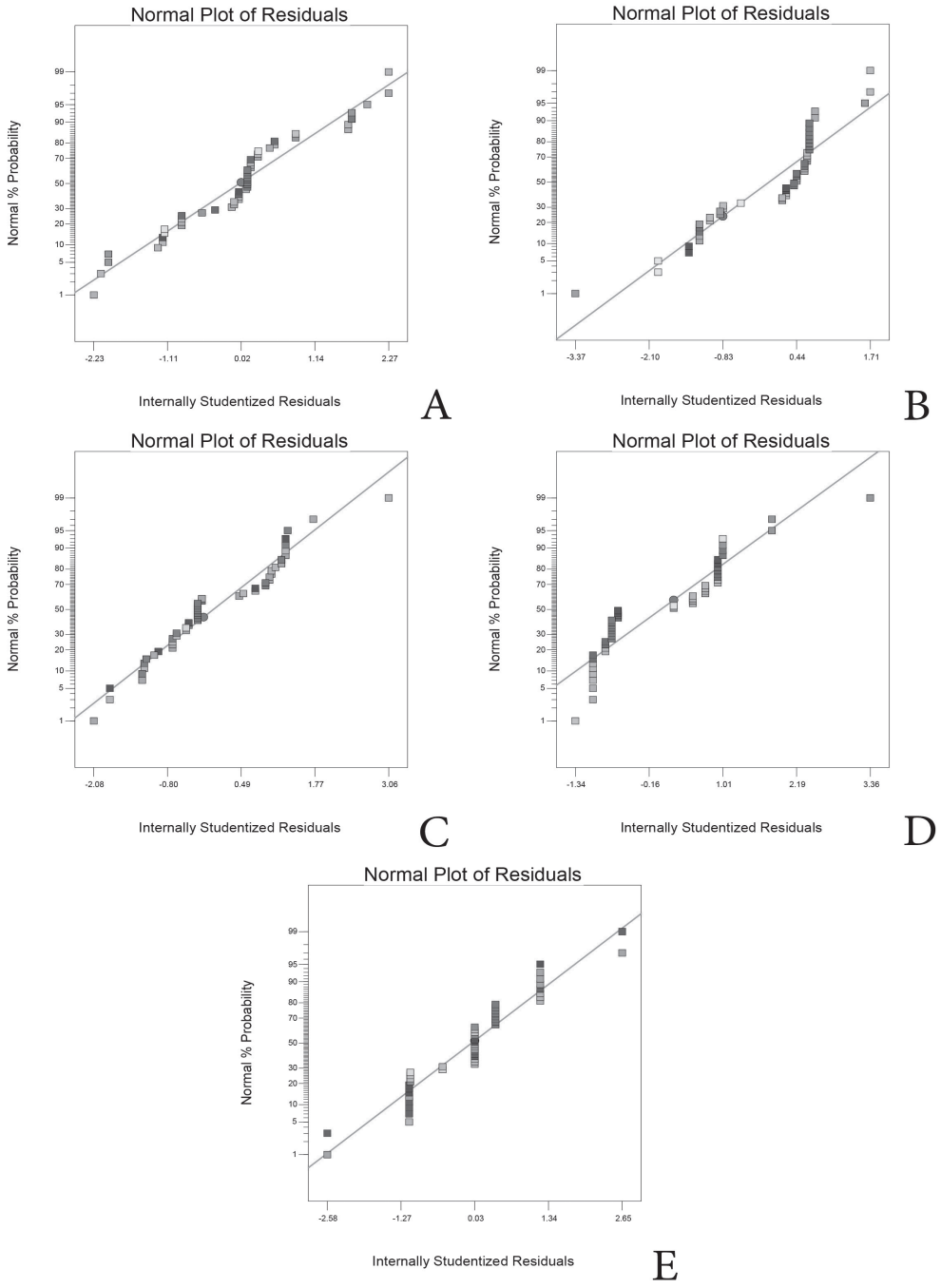

Fig. 6. Normal plots of residuals for responses A: *S.aureus*; B: *S.mutans*; C: *X. campestris*; D: *P.aeruginosa*; E: *C.albicans*

substrate concentration utilization and bioactive metabolite production with fermentation time yields μ_{max}, γ, and α value respectively. Ushakiranmayi *et al.* (16) also reported the similar attempt in process parameter evaluation. Model-derived biokinetic parameters are tabulated in Table 5 and very high determination coefficient (R^2) values (>0.99) for Logistic (L), Logistic Incorporated Leudeking-Piret (LILP), and Logistic Incorporated Modified Leudeking-Piret (LIMLP) model parameters to the experimental data obtained reveals good precision of the chosen models. Table 6 also confirms that the zones of inhibition from agar diffusion tests are almost similar to model predicted values.

Discussion

CCD of RSM is a mathematical and statistical method that finds application in analysing and evaluating the interaction of various parameters affecting the process. The advantage of RSM is the reduction in the number of experiments, improved statistical interpretation possibilities and the reduced time requirements for the overall analysis (24). CCD has been adapted and contemplated to be a very decisive statistical experimental design tool in optimization (25). The optimal conditions for the production of bioactive compound by VSM 13 were found to be time of incubation (10 days), pH (8), Temperature (35ºC), Concentration of fructose (1.5%) and Concentration of yeast extract (1%). Activity of the bioactive compound against the responses (zone of inhibition represented) was found to be 24 mm for *S. aureus*, 22 mm for *S. mutans*, 21 mm for *X. campestris*, 24 mm for *P. aeruginosa* and 25 mm for *C. albicans.*

Regression analysis was employed to fit the empirical model with the generated response variable data. Regression coefficients were determined with the least square method to develop second order quadratic polynomial models (26). From equation, the coefficient of one factor signifies the effect of individual while the coefficient which has two factors and second order signifies the interaction between them and their fourth route effect. The suffix symbols positive or negative (+/-) signify the synergy and antagonistic effects, where the positive stands for synergistic effect and the negative stands for antagonistic effect (27).

Adequacy of the model check is essential to check the model fitted to confirm that it delivers an accepted approximation to the actual system hence the ANOVA variance is applied. ANOVA of

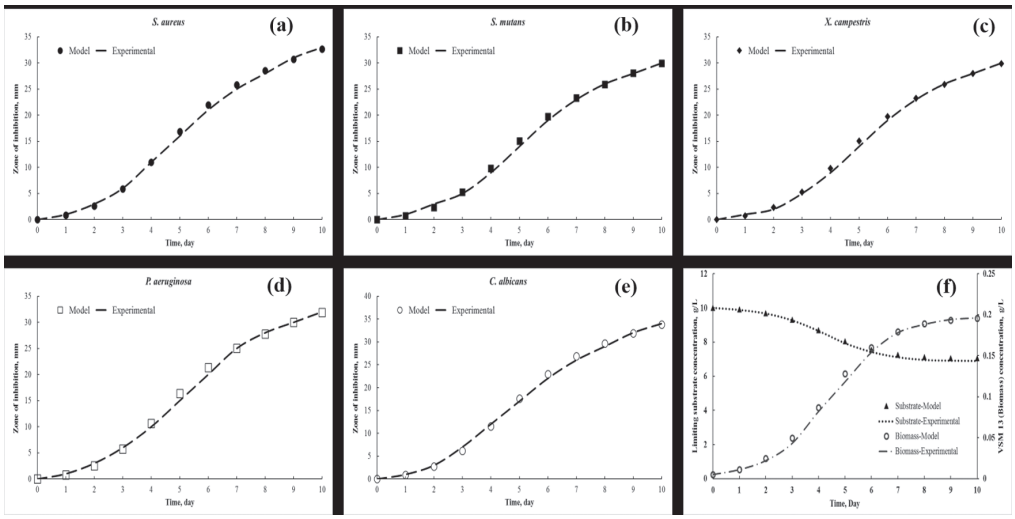

Fig.7. Comparison of experimental and model predicted kinetics (a)-(e): for zone of inhibition (mm); (f): for biomass growth (g/L), substrate utilization(g/L).

the model was analysed in order to determine the significance of the fitness of the quadratic model as well as the significance of the independent process variables and the interactive effects of process variables against the five responses (*S. aureus*, *S .mutans, X. campestris, P. aeruginosa and C. albicans*) whose effect is measured as zone of inhibition in mm. The ANOVA (F Value) and *p*- value are the key factors to estimate the coefficients of the model and to analyse the significance of each process variable and to study the interactive strength of each parameter (28). As per the Sequential Model Sum of Squares of all the 5 responses, the quadratic model was significant with (p-value <0.0001) for responses. ANOVA analysis showed that the confidence level of the model was greater than 95%. The model with the *p*- value less than 0.05 was considered statistically significant which implies that the model was acceptable for this experiment (29). Lower the value of *p* which narrates interactive effects between the variables and represents the importance of these variables in the model (30).

The lack of fit test values of quadratic models all the responses was found to be insignificant, which indicate that the model is highly significant. The goodness of the model fit of the regression analysis could be measured by the coefficient of determination (R^2) which indicates that the estimated model fits the experimental data satisfactorily. Statistical regression analysis of the equation shows that the R^2 for all the responses was found to be 0.9. The R^2 value shows that the model is significant predicting the response and explaining more than 95% of the variability in the production of the bioactive compound and its effect against the five responses. R^2 scale range for 0.1 and the value close to 1.0 signifies the fitness of the model (29). The model dependability is explained from the obtained values of the coefficient of determination (R^2) of the responses. The higher the R^2 values the more precise is the prediction responses based on the experimental data. These values indicate that the prediction of the experimental data obtained is satisfactory and can be used effectively to predict the responses. The theoretical value of the adj R^2 and the predicted R^2 of all the five responses in the model were given in Table 4 which indicate that the model is good fit.

Coefficient of Variation (CV) of the model indicates the degree of precision, flexibility and reliability of the experiment. In general the higher the value of CV the lower is the reliability of the experiment. In the present investigation the CV of all the five responses which are very low reveals better precision, flexibility and reliability of the experiment (31-32). Adequate precision measures the signal to noise ratio and the value greater than 4 indicate that the signal is adequate and the model can be used to navigate the design space (33). In addition the adequate precision correlates the range of the predicted values at the points of design to the average prediction error (34). Regression equation and coefficient of determination were evaluated to test the model fit (35).

3D plots determine the interaction among variables and the optimum level of each variable for maximum response. Response surface 3D plots show the interactions of the two independent variables where the other variable is fixed at zero and values obtained from the 3D plots were similar to optimum values reported (36). The values obtained from authentication were in good agreement with the optimization values showing the accuracy of the experiments carried out.

Adequacy of the model was analysed by the distribution of the residuals by determining whether the residuals followed a normal distribution. Residuals are the deviation between predicted and actual values and should follow the normal distribution if the experimental errors were random (37). Initially the residuals were normalized with respect to their standard deviations (studentized). Formation of the straight line indicates that the normal distribution is followed by studentized residuals (39). If the residuals do not follow a normal distribution an “S” shaped curve is obtained and this type of curve often results from the use of incorrect model and

Table 1. Experimental range of factors studied using CCD in terms of coded and actual factors

Factors	Symbols	Actual levels of coded factors		
		-1 (Low)	*0* (Middle)	*+1* (High)
Time of incubation (days)	*A*	9	10	11
pH	*B*	7	8	9
Temperature (^{0}C)	*C*	30	35	40
Concentration of Fructose (%w/v)	*D*	1	1.5	2
Concentration of Yeast Extract (%w/v)	*E*	0.5	1	1.5

Table 3 Sequential model fitting for all the responses (in terms of inhibition zone from the bioactive metabolite produced by the strain)

Model parameter	*Staphylococcus aureus*	*Streptococcus mutans*	*Xanthomonas campestris*	*Pseudomonas aeruginosa*	*Candida albicans*
***Sequential model sum of squares*- Quadratic vs 2FI (suggested)**					
Sum of squares	419.69	342.86	261.02	529.00	825.78
d.f.[a]	5	5	5	5	5
Mean square	83.94	68.57	52.20	105.80	165.16
F-value	387.42	1321.19	610.62	2472.83	2048.26
p-value (Prob > F)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
***Lack of ût tests*- Quadratic (suggested)**					
Sum of squares	2.34	1.51	2.48	1.24	2.34
d.f.[a]	22	22	22	22	22
Mean square	0.11	0.068	0.11	0.056	0.11
F-value	—-	—-	—-	—-	—-
p-value (Prob > F)	——	—-	—-	—-	—-
***Model summary statistics*- Quadratic (suggested)**					
Std. Dev.	0.28	0.23	0.29	0.21	0.28
R^2	0.9949	0.9961	0.9920	0.9978	0.9973
Adjusted R^2	0.9914	0.9933	0.9865	0.9963	0.9954
Predicted R^2	0.9809	009864	0.9899	0.9926	0.9899

d.f.- degrees of freedom

Table 4 ANOVA variance to test the adequacy of the model

Statistics	**Response**				
	Staphylococcus aureus	*Streptococcus mutans*	*Xanthomonas campestris*	*Pseudomonas aeruginosa*	*Candida albicans*
R^2	0.9949	0.9961	0.9920	0.9978	0.9973
Adj-R^2	0.9914	0.9933	0.9865	0.9963	0.9954
Pred- R^2	0.9809	0.9864	0.9699	0.9926	0.9899
AdequatePrecession	48.748	53.489	38.733	69.297	60.491
CV %	1.46	1.28	1.67	1.09	1.53

Table 2 Central composite factor experimental design along with experimental and predicted values

Run	*A*- Time (days)	*B*- pH	*C*- Temperature(^{0}C)	*D*- (%w/v)	*E*- [Yeast Fructose]	*Streptococcus* extract] (%w/v)	*aureus*	*Staphylococcus mutans*		*Xanthomonas campestris*		*Pseudomonas aeruginosa*		*Candida albicans*	
						Actual	RSM	Actual	RSM	Actual	RSM	Actual	RSM	Actual	RSM
1	11.00	7.00	40.00	1.00	1.50	19.00	18.94	18.00	17.90	18.00	17.79	18.00	18.16	17.00	16.99
2	11.00	9.00	40.00	2.00	0.50	19.00	19.18	18.00	17.90	18.00	18.15	18.00	18.16	17.00	17.23
3	11.00	7.00	30.00	1.00	1.50	18.00	18.18	17.00	17.20	17.00	17.15	18.00	17.92	17.00	16.76
4	11.00	7.00	40.00	2.00	0.50	19.00	18.54	17.00	17.20	17.00	17.26	18.00	17.92	17.00	16.99
5	11.00	9.00	40.00	2.00	1.50	19.00	18.61	17.00	16.88	17.00	17.43	18.00	17.89	17.00	16.76
6	11.00	7.00	40.00	2.00	1.50	19.00	18.97	17.00	16.88	18.00	17.80	18.00	17.89	17.00	16.99
7	10.00	8.00	35.00	1.50	1.00	18.00	17.97	16.00	15.93	17.00	16.80	17.00	17.16	16.00	16.52
8	11.00	9.00	30.00	1.00	0.50	18.00	18.45	16.00	15.93	17.00	16.91	17.00	17.16	17.00	16.76
9	10.00	8.00	35.00	1.50	1.00	18.00	17.89	17.00	16.91	16.00	15.89	17.00	17.16	15.00	15.23
10	10.00	7.00	35.00	1.50	1.00	18.00	18.00	17.00	16.91	16.00	16.25	17.00	17.16	16.00	15.46
11	11.00	9.00	30.00	2.00	1.50	17.00	17.00	16.00	15.96	15.00	15.25	17.00	16.92	15.00	14.99
12	9.00	9.00	40.00	2.00	0.50	17.00	17.24	16.00	15.96	16.00	15.36	17.00	16.92	15.00	15.23
13	9.00	7.00	40.00	2.00	1.50	17.00	17.43	16.00	16.13	16.00	15.79	17.00	16.89	15.00	14.99
14	10.00	8.00	35.00	1.50	1.00	18.00	17.67	16.00	16.13	16.00	16.15	17.00	16.89	15.00	15.23
15	9.00	9.00	40.00	1.00	1.50	17.00	16.67	15.00	14.94	15.00	15.15	16.00	16.16	15.00	14.76
16	10.00	9.00	35.00	1.50	1.00	17.00	17.03	15.00	14.94	15.00	15.26	16.00	16.16	15.00	14.99
17	9.00	7.00	30.00	1.00	1.50	18.00	17.91	16.00	16.17	16.00	16.38	17.00	16.86	16.00	15.99
18	11.00	7.00	30.00	1.00	0.50	18.00	18.02	16.00	16.17	17.00	16.74	17.00	16.86	16.00	16.23
19	11.00	7.00	40.00	1.00	0.50	17.00	17.02	16.00	15.72	16.00	15.74	17.00	17.13	16.00	15.76
20	11.00	9.00	30.00	1.00	1.50	17.00	17.25	16.00	15.72	16.00	15.85	17.00	17.13	16.00	15.99
21	11.00	7.00	30.00	2.00	0.50	17.00	16.94	16.00	15.90	16.00	15.77	16.00	16.10	16.00	15.76
22	11.00	9.00	40.00	1.00	1.50	17.00	17.18	16.00	15.90	16.00	16.13	16.00	16.10	16.00	15.99
23	10.00	8.00	35.00	1.50	1.00	16.00	16.18	15.00	15.20	15.00	15.13	16.00	15.86	15.00	15.52
24	9.00	7.00	40.00	1.00	1.50	17.00	16.54	15.00	15.20	15.00	15.24	16.00	15.86	16.00	15.76
25	11.00	9.00	40.00	1.00	0.50	17.00	16.98	15.00	14.93	15.00	14.73	16.00	15.86	14.00	14.23
26	9.00	9.00	30.00	1.00	1.50	17.00	16.97	15.00	14.93	15.00	15.09	16.00	15.86	15.00	14.46
27	11.00	7.00	30.00	2.00	1.50	16.00	15.97	14.00	14.23	14.00	14.09	16.00	16.13	14.00	13.99
28	9.00	7.00	40.00	2.00	0.50	16.00	16.08	14.00	14.23	14.00	14.20	16.00	16.13	14.00	14.23
29	10.00	8.00	35.00	1.00	1.00	16.00	15.89	15.00	14.91	14.00	14.38	15.00	15.10	14.00	13.99
30	9.00	8.00	35.00	1.50	1.00	16.00	16.00	15.00	14.91	15.00	14.74	15.00	15.10	14.00	14.23
31	9.00	7.00	30.00	2.00	0.50	15.00	15.00	14.00	13.96	14.00	13.74	15.00	14.86	14.00	13.76
32	11.00	9.00	30.00	2.00	0.50	15.00	15.24	14.00	13.96	14.00	13.85	15.00	14.86	14.00	13.99

33	9.00	9.00	30.00	2.00	0.50	23.00	23.41	21.00	21.14	21.00	20.81	24.00	23.85	24.00	23.99
34	9.00	9.00	30.00	2.00	1.50	24.00	23.65	21.00	21.14	21.00	21.04	24.00	23.85	24.00	24.23
35	10.00	8.00	35.00	1.50	1.00	23.00	23.41	21.00	21.56	21.00	20.81	23.00	22.97	23.00	23.23
36	10.00	8.00	35.00	2.00	1.00	23.00	22.65	21.00	20.73	20.00	20.04	23.00	22.73	23.00	22.99
37	11.00	8.00	35.00	1.50	1.00	23.00	22.83	21.00	20.97	20.00	20.10	23.00	22.73	23.00	23.23
38	10.00	8.00	35.00	1.50	1.00	22.00	22.24	20.00	20.32	20.00	19.75	22.00	21.97	23.00	22.99
39	9.00	9.00	30.00	1.00	0.50	23.00	23.12	21.00	21.20	21.00	20.75	23.00	22.85	24.00	23.99
40	9.00	7.00	30.00	1.00	0.50	22.00	21.94	20.00	20.09	19.00	19.10	22.00	21.85	22.00	22.23
41	10.00	8.00	40.00	1.50	1.00	22.00	22.24	20.00	20.32	20.00	19.63	23.00	22.50	22.00	22.11
42	9.00	7.00	30.00	2.00	1.50	21.00	20.83	19.00	18.97	18.00	18.22	21.00	21.20	21.00	21.11
43	10.00	8.00	35.00	1.50	1.00	24.00	23.97	22.00	21.86	21.00	21.08	24.00	24.15	25.00	24.89
44	9.00	9.00	40.00	2.00	1.50	24.00	23.97	22.00	21.86	21.00	21.08	24.00	24.15	25.00	24.89
45	10.00	8.00	35.00	1.50	1.00	24.00	23.97	22.00	21.86	21.00	21.08	24.00	24.15	25.00	24.89
46	9.00	9.00	40.00	1.00	0.50	24.00	23.97	22.00	21.86	21.00	21.08	24.00	24.15	25.00	24.89
47	10.00	8.00	35.00	1.50	1.50	24.00	23.97	22.00	21.86	21.00	21.08	24.00	24.15	25.00	24.89
48	10.00	8.00	35.00	1.50	0.50	24.00	23.97	22.00	21.86	21.00	21.08	24.00	24.15	25.00	24.89
49	9.00	7.00	40.00	1.00	0.50	24.00	23.97	22.00	21.86	21.00	21.08	24.00	24.15	25.00	24.89
50	10.00	8.00	30.00	1.50	1.00	24.00	23.97	22.00	21.86	21.00	21.08	24.00	24.15	25.00	24.89

an additional transformation of the response is required (39) (Fig. 6). The formation of the straight line indicates that the experiment is in agreement.

Logistic (L) model parameters, μ_{max}, X_0 and X_{max} were calculated from growth kinetic profile of *N. trehalosi* VSM 13 using experimental data. *N. trehalosi* VSM 13 is more growth associated than non-growth associated as growth associated parameter (α) value is higher than non-growth associated product parameter (β) from LILP model fitting. The simulated LIMLP model parameters, γ and η, are in good agreement with the experimental values, implies that this model is more appropriate to represent the limiting substrate utilization kinetics in bioactive metabolite production by *N. trehalosi* VSM 13. Further, results in Table 6 indicate that the zones of inhibition from agar diffusion tests are almost similar to model predicted values. All these results, confirm that the unstructured models provided a better approximation of kinetic profiles of bioactive metabolite production by *N. trehalosi* VSM 13 in submerged shake flask fermentations.

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Conflict of interest

All authors declare that there is no conflict of interest.

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Table 5 Estimated kinetic parameters using L, LILP, LIMLP model equations

Kinetic Parameters	*S. aureus*	*S. mutans*	*X. campestris*	*P. aeruginosa*	*C.albicans*
Logistic (L) Model Parameters					
μ_{max} (d^{-1})	0.853				
R^2	0.996				
X_0 (g/L)	0.005				
X_m (g/L)	0.197				
Logistics incorporated Modified Luedeking-Piret (LIMLP) Model parameters					
ã (g.S/g.X)	16.383				
R^2	0.994				
ç (g.S/(g.X.d))	0.101				
Logistics incorporated Luedeking-Piret (LILP) Model parameters					
á (mm/g.X)	120.45	106.05	105.95	116.42	126.7
R^2	0.997	0.998	0.999	0.997	0.994
â (mm/(g.X.d))	8.658				

Table 6 Comparison of zones of inhibition (mm) from shake-flask experiments and from model

Maximum	*S. aureus*	*S. mutans*	*X. campestris*	*P. aeruginosa*	*C. albicans*
Zone of Inhibition (mm)					
Experimental	33	30	30	32	34
Model fitted	32.673	29.929	29.91	31.905	33.864

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In-silico Prediction of T and B cell Epitopes in the Evolutionary Conserved Pathway of Glycolysis for Human Pathogens: *Coccidiodes immitis, Histoplasma capsulatum and Pneumocystis carnii*

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Abstract

Fungal diseases are amongst the emerging diseases to which humans are most susceptible pertaining to the present day life style. New drugs are being made but at a slow pace, not matching the resistance developing capacity of the fungal pathogens. Therefore, it is important to choose new drug targets peculiar to fungi but absent in humans.A common practice till date has been to use the virulence proteins in order to devise medicines against micro-organisms. However, we have used *in-silico* techniques to analyze enzymes involved in the evolutionary conserved pathway of glycolysis which is the most primitive pathway for ATP production in aerobic as well as anaerobic organisms. Therefore, on successfully targeting these enzymes microorganism can be killed. We have chosen 3 fungal pathogens viz.*Coccidiodes immitis, Histoplasma capsulatum and Pneumocystis carnii* that cause serious diseases in human beings and their effect on the morbidity and mortality of humans has been substantial. MEME-Motif discovery tool was used to devise a single motif sequence per glycolytic enzyme from the three fungi. Further, online servers such as ABCpred, Bcepred, BepiPred and Tools from Immunomedicine group have been used in order to predict the linear epitopes from the motif sequences of the different glycolytic enzymes. Analysis of these epitopes was performed through PepCalc and NetSurfP in terms of parameters such as hydrophilicity, coil forming residues and exposed residues. Analysis of ten proteins of glycolysis from each fungus reveals the regions that can elicit an immunological response. This study most importantly projects aldolase to be an enzyme of importance which in future can be used as a potential vaccine target for these three fungi.

Keywords: *In-silico*, Epitope, Motif, Glycolysis, Fungi.

Introduction

In the recent past, there has been observed a massive increase in the human pathogenic diseases. The reason for the same can be attributed to the changing lifestyle as well as the modern day technologies (1). In-spite of the tremendous advancement of the medical facilities, these diseases are likely to follow an exponential expansion in the near future, reason being the fast pace at which the disease causing microorganisms are gaining resistance to the currently available drugs (1).

Therefore, the need of the hour is to find out new drug targets against these pathogens and develop suitable therapeutics for the same. The situation is graver when it comes to anti-mycotic drugs. The number of efficient drugs against the pathogenic fungi is very limited and those that are available are not free from side effects (2). The reason for the same is that fungi are eukaryotic organisms and have a lot in common with the human body cells. Therefore, it is difficult to devise suitable targets that would only be present in fungi and not in humans. Most of the research in this area is focused on identifying the various virulence factors and then to target the same with a suitable

therapeutic agent (2). But, in the current study, the authors have tried using a completely different approach to counter three fungal pathogens (*Coccidiodes immitis*, *Histoplasma capsulatum and Pneumocystis carnii*)by using the enzymes present in the evolutionary conserved pathway of glycolysis to serve as suitable antifungal drug targets. The reason behind choosing these fungal pathogensis that in the recent past a mass increase has been observed in the morbidity and mortality amongst the humans and the animals due to them (3). It is thus important to understand the pathology of these fungal organisms before stepping towards the immunoinformatic part of the manuscript.

Coccidiodes immitis*: Coccidiodes immitis* is a fungal pathogen responsible for causing valley fever or coccidioidomycosis. It is an endemic resident to the soil of south western United States, some parts of Mexico and Central and South America. It has a high prevalence due to its mode of infection. Its spores are present in the air and inhalation causes them to enter the lungs where the primary infection takes place (4). The general symptoms of the disease make it very difficult to distinguish from the other pulmonary diseases. Symptoms include fever, sore throat, cough, rashes on upper body and legs, night sweats, joint pain, headache, fatigue, and pleuritic chest pain etc. Generally, the body is able to ward off the infection by itself but in some cases medication is needed. A 3-6 months of fluconazole or any other antifungal administration will be enough to treat the disease. A recent study portraits the fact that 3089 coccidioidomycosis associated deaths have occurred in United States from 1990 to 2008 which accounts for about 200 deaths per year. This emphasizes the need of a suitable medicine to treat the fungus (http://www.cdc.gov/fungal/diseases/coccidioidomycosis/statistics.html; http://emedicine.medscape.com/article/215978-overview).

Histoplasma capsulatum: *Histoplasma capsulatum* is the causal organism of histoplasmosis. Large amounts of bat and bird droppings in a particular region make the soil and the area endemic to *Histoplasma*. It has been found in parts of America, Africa, Australia and Asia. Histoplasmosis generally occurs due to inhalation of aerial spores. Fever, cough, body ache, head ache, chills etc are some of the common symptoms of histoplasmosis (5). Itraconazoleis a drug of important therapeutic value in case of this disease and the course of the treatment may last from 3 months to about a year depending upon the severity of the symptoms. A study performed on patients hospitalized due to histoplasmosis reports that a mortality rate of 5% and 8% is associated with children and adults respectively (6).

Pneumocystis carnii: *Pneumocystis carnii* is the causal organism of a serious illness called Pneumocystis pneumonia (PCP). PCP can cause severe illness in people having weakened immune system. People with HIV/AIDS are more prone to this disease. The symptoms of this disease include fever, rapid breathing, cough etc. (https://www.nlm.nih.gov/medlineplus/ency/article/000671.htm). The best therapeutic option for this disease is Trimethoprim Sulfamethoxazole (TMP-SMX) which should be taken for three weeks. *Pneumocystic* pneumonia can cause lung failure hence is a life threatening disease. Nevertheless, the use of corticosteroids in people suffering from AIDS has decreased the amount of deaths (7). Amongst immunocompromised patients the mortality rates range from 5-40% in those who receive treatment whereas approaches 100% without treatment (http://www.cdc.gov/fungal/diseases/pneumocystis-pneumonia/statistics.html).

All of the above described fungi utilize glycolysis as the source for energy, precursor molecules and reducing power (in the form of NADPH). Glycolysis in general is a process in which glucose is converted into pyruvic acid by some enzyme catalyzed reactions. The utility of this pathway can be proved by the fact that it is ubiquitous in nature. Although this pathway has been deciphered decades before but very little is known about its regulation and control. This is the backbone of respiration and is of utmost

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importance for survival of both prokaryotes and eukaryotes.

There are slight variations amongst animals and fungi with respect to the enzymes that are utilized in glycolysis. For instance, two classes of aldolases have been found in nature. Class I aldolase is primarily present in plants and animals whereas class II aldolase is present in bacteria and fungi. Both aldolases utilize different reaction mechanisms, aldolase I requires Schiffs base as a reaction intermediate for the cleavage of fructose 1,6 bisphosphate whereas aldolase II makes use of Zn^{2+} in its active site (https://www.ebi.ac.uk/interpro/potm/2004_2/Page2.htm) for the same. Therefore, the authors have chosen this pathway for the identification of epitopes which can be targeted in order to devise suitable medicine against *C. immitis, H. capsulatum* and *P.carnii.*

Materials and Methods

Retrieval of the sequences of the respective genes from NCBI: The sequences of all the genes of the enzymes of glycolysis (Hexokinase, Phosphoglucoseisomerase, Phosphofructokinase, Fructose-bisphosphate-aldolase, Triosephosphate isomerase, Glyceraldehyde 3-phosphate dehydrogenase, Phosphoglycerate kinase, Phosphoglyceratemutase, Enolase and Pyruvate kinase) from the three organisms viz*Coccidiodes immitis, Histoplasma capsulatum and Pneumocystis carnii* have been retrieved from NCBI.

Discovery of the motif through MEME-Motif discovery tool: The sequences obtained from NCBI were subjected to the MEME- Motif discovery tool (http://meme-suite.org/). A single conserved sequence for all the three organisms for each gene was found out. The same process was repeated for all the enzymes of glycolysis.

***Mapping linear IgE binding epitopes*:** ABCpred (8), Bcepred (9), BepiPred (10) and Tools from Immunomedicine (11) group web servers were employed to predict linear B-cell epitopes. The web servers utilize the sequence of the protein as input and provide the possible epitopes as output based on the concepts of artificial neural networks, support vector machine, hydrophilicity scale with a hidden markov model, amino acid propensity scales of hydrophilicity, surface probability, antigenic index and flexibility (12).

***Property predicting softwares for the identified epitopes*:** Identified epitopes were analyzed with the help of 2 property predicting softwares viz. PepCalc (http://pepcalc.com/) and NetSurfP (http://www.cbs.dtu.dk/services/NetSurfP/). PepCalc analysed the epitopic sequences on the basis of parameters such as hydrophilic residues and polar residues whereas NetSurfP analyzed the epitope on the basis of the number of coil forming and the exposed residues.

Results

Retrieved sequences (Table-1) of different genes of glycolysis from *C.immitis, H.capsulatum and P. carnii* were subjected to MEME-Motif discovery tool and the obtained motif sequences of various glycolytic genes (Table 2) were used for epitope prediction.The individual results obtained from ABCpred, Bcepred, BepiPred and Tools from Immunomedicine group server have been provided in table 3, 4,5 and 6respectively. A single common epitope with the highest immunogenicity was chosen with the help of the mentioned webservers (Table-7).

Further, the identified epitopes were analyzed and the results of the same have been depicted in fig.1, which showed that the epitopes are high in the hydrophilic residues, coils and also

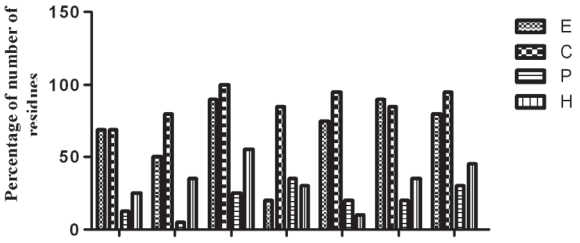

Fig. 1. A graph depicting the percentage of number of residues forming exposed (E), coiled(C), polar (P) and hydrophilic(H) regions in different glycolytic proteins. A:Hexokinase, B:Phosphofructokinase, C:Aldolase, D:Triosephosphateisomerase, E:Phosphoglycerate kinase, F: Enolase,G:Pyruvate kinase.

Table 1. Glycolytic genes and their respective IDs.

Glycolytic genes	*C. immitis*	*H.capsulatum*	*P. carnii*	*Homo sapiens*
Hexokinase	EAS35711.2	EDN07130.1	XP_007874379.1	AAC00173.1
Phosphoglucoisomerase	EAS29410.2	EDN04536.1	XP_007875677.1	P06744.4
Phosphofructokinase	EAS27971.2	EGC45399.1	XP_007872216.1	AAA60068.1
Fructose bisphosphatealdolase	3PM6	EGC40786.1	XP_007875432.1	P05062.2
Triose phosphate isomerase	P0CL22.1	EGC44423.1	XP_007874541.1	CAA49379.1
Glyceraldehyde-3-phosphate dehydrogenase	Q1DTF9.1	AAG33369.1	XP_007873850.1	CAA25833.1
Phosphpoglycerate kinase	EJB11848.1	EDN06855.1	XP_007874060.1	AAA60079.1
Phosphoglyceratemutase	EAS32175.2	EGC49880.1	XP_007875232.1	AAA60073.1
Enolase	EAS28926.2	EGC42819.1	XP_007874896.1	AAB59554.1
Pyruvate kinase	EAS29628.2	EGC42746.1	XP_007874297.1	AAA60104.1

Table 2. Motif sequences of the glycolytic genes obtained through the MEME-Motif discovery tool.

Name of the gene	Motif sequences
Hexokinase	INDTTGTLIASAYTDPEMRIGCIFGTGVNAAYMENAGSIPKIAHYN LPPDMPIAINCEY GAFDNERVVL
Phosphoglucoseisomerase	EVTKFGIDKRNMFGFESWVGGRYSVWSAIGLSVALYIGFDNFH QFLAGAHAMDKHFRETP LEQNIPVLGG
Phosphofructokinase	HRICEAVDEVFDTAASHQRGFVIEVMGRHCGWLALMSAISTGA DWLFIPE MPPRDGWEDD
Aldolase	SNIELTKRYLQRIAPMKQWLEMEIGITGGEEDGINNEEVSNNKL YTRPEDVF DVYSALSAISPYFSIAAAFGNVH
Triosephosphate isomerase	EHLRDDICVSAQNVYNSPPGPYTGEITVEQLKDAKILWTIV GHSERRIYFNESNQFIALK TKRALENGMS
Glyceraldehyde 3-phosphate dehydrogenase	ETYKSDIKVLSNASCTTNCLAPLAKVVNDNFGLVEGLMTTVHSY TATQKTVDGPSSKDWR GGRAAAQNII
Phosphoglycerate kinase	RKGLTALGDIYINDAFGTAHRAHSSMVGVNLPQRAAGFLVKK ELEYFAKALENPTRPFLA ILGGSKVSDK
Phosphoglyceratemutase	Common motif not found between the chosen sequences
Enolase	QACKLAQENGWGVMVSHRSGETEDTFIADLVVGLCTGQIKTGA PCRSERLAKYNQLMRIE EELGDEARFA
Pyruvate kinase	DEILDQADGVMVARGDLGIEIPAPKVFIAQKMMIAKCNIKGK PVICATQMLESMTYNPRP TRAEVSDVAN

Table 3. Epitopes for each glycolytic gene as predicted by ABCpred webserver along with their respective scores.

Glycolytic genes	Identified epitopes	Score
Hexokinase	DMPIAINCEYGAFDNE	0.95
	IGCIFGTGVNAAYMEN	0.91
	IASAYTDPEMRIGCIF	0.88
	AHYNLPPDMPIAINCE	0.84
	YMENAGSIPKIAHYNL	0.71
	TGVNAAYMENAGSIPK	0.69
Phosphoglucoseisomerase	KFGIDKRNMFGFESWV	0.83
	GFESWVGGRYSVWSAI	0.80
	YSVWSAIGLSVALYIG	0.70
	KHFRETPLEQNIPVLG	0.69
	GLSVALYIGFDNFHQF	0.69
	GAHAMDKHFRETPLEQ	0.64
	YIGFDNFHQFLAGAHA	0.56
Phosphofructokinase	AISTGADWLFIPEMPP	0.84
	HQRGFVIEVMGRHCGW	0.79
	WLFIPEMPPRDGWEDD	0.77
	GRHCGWLALMSAISTG	0.73
	RICEAVDEVFDTAASH	0.60
Aldolase	DEVFDTAASHQRGFVI	0.58
	MEIGITGGEEDGINNE	0.93
	EDGINNEEVSNNKLYT	0.82
	NNKLYTRPEDVFDVYS	0.80
	TKRYLQRIAPMKQWLE	0.64
	YSALSAISPYFSIAAA	0.64
	RIAPMKQWLEMEIGIT	0.51
Triosephosphate isomerase	LWTIVGHSERRIYFNE	0.95
	TGEITVEQLKDAKILW	0.88
	DICVSAQNVYNSPPGP	0.83
	ERRIYFNESNQFIALK	0.74
Glyceraldehyde 3-phosphate dehydrogenase	QKTVDGPSSKDWRGGR	0.90
	PSSKDWRGGRAAAQNI	0.88
	TYKSDIKVLSNASCTT	0.85
	LVEGLMTTVHSYTATQ	0.83
	ASCTTNCLAPLAKVVN	0.75
	CLAPLAKVVNDNFGLV	0.58
Phosphoglycerate kinase	GFLVKKELEYFAKALE	0.82
	FGTAHRAHSSMVGVNL	0.82
	PTRPFLAILGGSKVSD	0.71
	ELEYFAKALENPTRPF	0.64
	VGVNLPQRAAGFLVKK	0.56

Enolase	HRSGETEDTFIADLVV	0.92
	TGQIKTGAPCRSERLA	0.83
	ENGWGVMVSHRSGETE	0.76
	GAPCRSERLAKYNQLM	0.57
	ERLAKYNQLMRIEEEL	0.54
	DTFIADLVVGLCTGQI	0.53
	QLMRIEEELGDEARFA	0.52
Pyruvate kinase	GIEIPAPKVFIAQKMM	0.92
	EILDQADGVMVARGDL	0.82
	GVMVARGDLGIEIPAP	0.80
	PKVFIAQKMMIAKCNI	0.78
	ESMTYNPRPTRAEVSD	0.77
	MMIAKCNIKGKPVICA	0.72
	GKPVICATQMLESMTY	0.66

Table 4. Epitopes for each glycolytic gene as predicted by Bcepred webserver along with their respective scores.

Glycolytic genes	Identified epitopes	Score
Hexokinase	LINDTTGTLIASAYTDPEMR	0.805
Phosphoglucoseisomerase	None	
Phosphofructokinase	TGADWLFIPEMPPRDGWEDD	0.874
Aldolase	EIGITGGEEDGINNEEVSNN	0.999
Triosephosphateisomerase	VSAQNVYNSPPGPYTGEITV	0.998
Glyceraldehyde 3-phosphate dehydrogenase	None	-
Phosphoglycerate kinase	AHRAHSSMVGVNLPQRAAGF	0.789
Enolase	GLCTGQIKTGAPCRSERLAK	0.931
Pyruvate kinase	LESMTYNPRPTRAEVSDVAN	0.937

form the exposed regions. The maximum percentage of the residues forming the exposed regions i.e. 90% was in Aldolase and Enolase followed by Pyruvate Kinase (80%), Phosphoglycerate kinase (75%), Hexokinase (68.75%), Phosphofuctokinase (50%) and Triosephosphate isomerase (20%). Similarly, the highest percentage of the coil forming residues were also observed (100%) in Aldolase followed by Pyruvate kinase, Phosphoglycerate kinase, Enolase, Triosephosphate isomerase, Phosphofructokinase and Hexokinase with 95%, 95%, 85%, 85%, 80% and 68.75% respectively. Likewise, the maximum percentage of the residues forming the hydrophilic regions i.e. 55% was also in Aldolase followed by Pyruvate kinase (45%), Enolase (35%), Phsphofructokinase (35%), Triosephosphateisomerase (30%), Hexokinase (25%) and Phosphoglycerate kinase (10%).

Discussion

Three primary pathogenic fungi viz. Coccidiodes immitis, Histoplasma capsulatum and Pneumocystis carnii are responsible for increasing morbidity and mortality in the humans and animals. In the past some amount of work has been done in the field of epitope prediction and targeting with respect to non-glycolytic genes in the above

Table 5. Epitopes for each glycolytic gene as predicted by BepiPred webserver along with the score of each residue comprising the epitope.

Glycolytic genes	Identified epitopes along with the scores of each residue
Hexokinase	Y(0.802)T(0.966)D(1.040)P(0.618)E(0.529)M(0.352) M(0.467)E(0.530)N(0.478)A(0.557)G(0.755)S(0.759)I(0.575)P(0.385) N(0.596)L(0.751)P(0.879)P(0.689)D(0.854)M(0.383)P(0.514)
Phosphoglucoseisomerase	F(0.355)R(0.360)E(0.438)T(0.472)P(0.723)L(0.839)E(0.800)Q(0.472)
Phosphofructokinase	E(0.363)M(1.075)P(1.534)P(1.678)R(1.953)D(1.957)G(2.050) W(2.094) E (2.125)D(1.965)D(1.703)D(0.523)T(0.385)A(0.455)A (0.594)S(0.870) H(0.394)
Aldolase	G(0.534)I(0.826)T(1.041)G(1.148)G(1.533)E(1.328)E(1.698) D(1.681) G(1.692)I(1.626)N(1.258)N(1.106)E(0.994)E(1.005)V(1.271)S (1.220)N(1.186)N(1.091)
Triosephosphate isomerase	N(0.491)V(1.009)Y(1.350)N(1.760)S(1.913)P(1.924)P(2.284)G (2.458)P(2.443)Y(1.924)T(1.692)G(1.175)E(0.835)I(0.609)
Glyceraldehyde 3-phosphate dehydrogenase	Y(0.460)T(0.556)A(0.776)T(0.745)Q(1.006)K(1.308)T(1.422)V (1.690)D(1.842) G(1.847)P(1.965)S(1.690)S(1.889)K(1.706)D (1.626)W(1.552)R (1.312)G(1.048)G (0.936) R(0.814)A(1.030)A(0.614)
Phosphoglycerate kinase	G(0.516)T(0.390)A(0.294)H(0.032)R(0.547)L(0.481)E(0.954)N (1.011)P(0.781)T(0.529)R(0.539) K(0.801)V(1.172)S(1.224)D (1.197)K(1.088)
Enolase	I(0.663)K(0.793)T(0.824)G(0.916)A(0.846)P(1.098)C(1.028) R(0.674)R(0.6 30)S(1.056)G(1.495)E(1.533)T(1.280)E(0.948)D (0.669)T(0.556)E(0.630)L(0.721)G(1.018)D(0.611)E(0.456)
Pyruvate kinase	T(0.526)Y(1.023)N(1.205)P(1.363)R(1.482)P(1.562)T(1.511) R(1.408)A (1.367)E(1.0 18)V(0.812)S(0.722)D(0.562)V(0.633)A(0.514)N(0.691)

mentioned fungi. For instance, Antigen 2 (Ag2) has been identified as a major component of the mycelium and spherule based cell wall that can elicit T-cell responses in case of *Coccidioides* immune mice (13). Herr et al. (2007) (14) reported the presence of 6 epitopes (1P6:TRLTDF KCHCSKPELPGQIT; 1P7:HCSKPELPG QITPC VEEACP; 1P12: PIDIPPVDTTAAPE-PSETAE; 1P13: TTAAPEPSETAEPTAEPTEE; 1P15: PTEE PT AEPTAEPTAEPTHE and1P16: PTAEPTA EPTHEPTEEPTAV) in the Antigen 2 (Ag2)/Pra and the presence of 3 epitopes in (2P6: EKLTDF KCHCAKPELPGKIT; 2P13: DTRTPTQP PSTSPSAPQPTA; 2P14: PSTSPSAPQPTACI-PKRRRA) in PrP2 antigen. Similarly, Hurtgen et al (2012) (15) reported the presence of 2 antigens (P1: MRNSILLAATVLLGCTSAKVHL and P2: HVRALGQKYFGSLPSSQQQTV) in Pep1 antigen. In another study,6 antigenic epitopes of PRA viz. ARISVSNIV, GRPTASTPA, ALNLFVEAL, LVAAGLASA, FSHALJALV, AEPTHEPTE were identified so that the future therapeutics could be

Table 6. Epitopes for each glycolytic gene as predicted by Tools from Immunomedicine group webserver along with theaverage antigenic propensity score for each protein.

Hexokinase (Average antigenic propensity for this protein is 1.0173)	GTLIASA MRIGCIFG SIPKIAHYNL IAINCEY
Phosphoglucoseisomerase (Average antigenic propensity for this protein is 1.0226)	VGGRYSVWSAIGLSVALYIG NFHQFLAG
Phosphofructokinase (Average antigenic propensity for this protein is 1.0229)	CEAVDEVFDTAASHQRGFVIEVMG HCGWLALMS
Aldolase(Average antigenic propensity for this protein is 1.0075)	LTKRYLQR DVFDVYSALSAISPYFSIAAA
Triosephosphate isomerase(Average antigenic propensity for this protein is 1.0169)	RDDICVSAQNVYNSPPG KDAKILWTIVG QFIALKT
Glyceraldehyde 3-phosphate dehy drogenase (Average antigenic propensity for this protein is 1.0243)	SDIKVLSNA CTTNCLAPLAKVVNDNFGLVEGLMTTVHSY
Phosphoglyceratekinase(Average antigenic propensity for this protein is 1.0291)	HSSMVGVNLPQRAAGFLVKKELEYFAK TRPFLAILGG
Enolase(Average antigenic propensity for this protein is 1.0163)	WGVMVSH FIADLVVGLCTG KTGAPCRS
Pyruvate kinase(Average antigenic propensity for this protein is 1.0289)	DGVMVAR LGIEIPAPKVFI AKCNIKGKPVICAT

targeted against them (16). In case of *H. capsulatum*, epitopes have been reported on glycoproteins H,M and C antigens.They are common in major genera of dimorphic fungi and reported to be cross reactive galactomannan (17). A study made on the M antigen by using Jamenson-Wolf algorithm (Protean program, DNASTAR Inc, Madison, Wis., USA) reported that a large number of epitopes were found between amino acid 212 to 442 (18). Scheckelhoff and Deepe (2012) (19) observed the presence of an epitope (DGGQG) in the antigenic CDR3 fragment. Wang et al. (20) performed epitope mapping in *P. marneffei* and suggested that Mp1p, a cell wall mannoprotein can be used as a biomarker for its diagnosis and can also be targeted for therapeutic applications.

In the present study, the motif regions have been identified amongst all the three microorganisms for the proteins required in the glycolysis. And these motif sequences were further used for predicting the linear epitopes suggesting that aldolase amongst all the glycolytic enzymes can prove to be a fruitful

Table 7. Epitopes found from the motif sequences of the genes through ABCpred, Bcepred, BepiPred and Tools from Immunomedicine group.

Hexokinase	IASAYTDPEMRIGCIF
Phosphoglucoseisomerase	No epitope with suitable immunogenicity could be found
Phosphofructokinase	TGADWLFIPEMPPRDGWEDD
Aldolase	EIGITGGEEDGINNEEVSNN
Triosephosphate isomerase	VSAQNVYNSPPGPYTGEITV
Glyceraldehyde 3-phosphate dehydrogenase	No epitope with suitable immunogenicity could be found
Phosphoglycerate kinase	AHRAHSSMVGVNLPQRAAGF
Phosphoglyceratemutase	Common motif not found between the chosen sequences
Enolase	GLCTGQIKTGAPCRSERLAK
Pyruvate kinase	LESMTYNPRPTRAEVSDVAN

target for the future drugs against *C.immitis, H.capsulatum* and *P. carnii.* Mor et al. (21) suggested that aldolase can be used as a target antigen in case of Alzhiemer's disease.Similarly, in another study on cancer cells provides the insight that the aldolase is responsible for reduction in the cell proliferation by 90% utilizing a non-metabolic approach (22). Henceforth, aldolase is not only responsible for carrying out the glycolysis and production of ATP but it is also related to the cancer pathway in some way or the other. Furthermore, a study conducted by McCarthy et al. (23) on *Onchocerca volvulus* suggests thataldolase of this parasite can act as a vaccine candidate. Similarly, another study aiming to identify the target of the autoantibodies produced in patients suffering from hyperkinetic movement disorders (HMD) deciphered that glycolytic enzyme aldolase A was the target of those autoantibodies (24). Thus, from the above account it is clear that aldolase is not only useful in the glycolytic pathway but it has many roles in multiple areas of targeting.

Conclusion

A number of conclusions can be drawn from this study. First and the foremost is that glycolysis being the universal pathway can be targeted by the drugs for killing the micro-organisms. Secondly, a number of bioinformatics tools are available which can be used for determining the motif regions as well as the epitopic regions present in a sequence. Thirdly, the salient features of the epitopes are that they are rich in the residues which are hydrophilic in nature, are efficient in the formation of coils and also form the exposed regions. Fourthly, aldolase is most probable enzyme which can be used to determine epitopes and further usage as vaccine candidate against *Coccidiodes immitis, Histoplasma capsulatum* and *Pneumocystis carnii.*

Conflict of Interest

None

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Alpha Amylase Inhibitory Activity of Different Extract of *Terminalia arjuna* Bark

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Abstract

In India, medicinal plants are used by all sections of people either directly as folk remedies or in different indigenous system of medicine. The present study was based on the use of ethnomedicinal plant bark to evaluate the alpha amylase inhibitory activity. Bark of *Terminalia arjuna* was collected and air dried and soxhlet extracted by using standard methods for flavonoid, alkaloid, steroid and different solvents. These extracts obtained were then tested for the alpha amylase inhibitory activity using the chromogenic DNSA method and starch iodine min triplicates. Data were expressed as mean ± SEM (standard error of the mean). Data were analyzed by one-way analysis of variance and *P* values were considered significant at $P<0.05$. Among the tested extracts, methanol, free flavonoids and bound flavonoids extract exhibited significant alpha amylase inhibitory activity. Results of the present study revealed that methanol extracts of *T. arjuna* bark showed great antidiabetic potential and may be exploited for future antidiabetic drugs.

Key words: Bark, alpha Amylase, Antidiabetic, methanolic

Introduction

Diabetes is one of the common diseases found in both developed as well as developing countries and one of the estimation it has been found that 1/3 rd of diabetic person use some form of complementary and alternative medicine (1). Herbal products are very important ingredients which can be used in the development of drug (2). Now very less modern medicines are available to act as a remedy for various diseases, in such situation many bioactive compounds of medicinal plants are used as anti diabetic, chemotherapeutic, anti inflammatory and anti arthritic agents[3]. Plants produce secondary metabolites rich in flavonoids, phenolic compounds, alkaloids and steroids which help in prevention of various diseases (4). α-Amylase is a enzyme that catalyses the hydrolysis of internal linkage of α-1, 4-glycosidic bond in starch to yield products like glucose and maltose (5). Thus, inhibition of the alpha amylase enzyme in the digestive tract of humans is being considered to be effective in controlling diabetes by decreasing the absorption of glucose from starch (6).

Terminalia arjuna, traditionally has been used as a medicinal plant for the prevention heart diseases. It has been described as three humours viz., vata, pitta and kapha in Ayurveda (7). The tree is commonly known as Arjuna and belongs to family *Combretaceae*. Bark of T. arjuna has been used in the traditional system and reposted to contain different groups of chemical compounds like flavanoids, phenolics, and phyto sterols [8]. Arjunolic acid is an important compound (2, 3, 23-trihydroxyolean-12-en-28-oic acid) used as an antioxidant, antiallergic, and antiasthmatic (9).

The diabetes leads to increased risk of other complications such as cardio vascular disease, peripheral vascular disease complications. There are many agents available to treat diabetes, but

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the main disadvantages of these drugs are that they required to be given to patients for their whole life (10). As herbal plant products are used as medicine since ancient time by people of different countries for the treatment in diabetes mellitus. Hence, there is increasing emphasis on the use of plant products for the effective management of the disease. So far there are no reports showing the comparative alpha amylase inhibitory activity of polar, non polar, free flavonoids, bound flavonoids, alkaloid and steroid extract of *T arjuna* bark. Hence in the present study an attempt was made to compare the alpha amylase inhibitory activity of different extracts of the bark of *T. arjuna.*

Materials and Methods

***Collection of Plant Material*:** Bark of *Terminalia arjuna,* was collected from the eastern region of Rajasthan i.e. Jaipur. Plant was identified by the senior taxonomist of the Department of Botany, University of Rajasthan and Voucher specimen no: RUBL211458 was submitted to the Herbarium, Department of botany, University of Rajasthan.

***Preliminary phytochemical screening*:** The phytochemical analysis to test the presence of alkaloids, flavonoids, steroids in the powdered sample was carried out using standard methods (11-12).

Preparation of Extracts

***Flavonoid extraction*:** Bark of *Terminalia arjuna* was collected; shade dried, finely powdered and Subramanian & Nagarjan method was used for extraction of flavonoids (13). 100 grams of sample with 80 % of hot methanol was soxhlet extracted on a water bath for 24 h and filtered. The filtrate was re-extracted using separating funnel successively with petroleum ether in the first fraction and ethyl ether in the second fraction and ethyl acetate in the third fraction. As petroleum ether dissolves fatty substances in it, the fraction of it was discarded, where as fractions of ethyl ether and ethyl acetate were further analyzed for free and bound flavonoids respectively. The ethyl acetate sample was refluxed with 7% H_2SO_4 for two hours so that hydrolysis removed bounded sugars and again in separating funnel, the filtrate was refluxed with ethyl acetate. To neutralize the obtained filtrate it was washed with distilled water. Thus, the ethyl ether fraction contain free flavonoids and ethyl acetate fractions contains bound flavonoids were dried in vacuum and weighed. The obtained extracts were stored at 4°C.

***Alkaloids Extraction*:** Alkaloids were extracted from bark of *T. arjuna* by well established method [14]. Hundred grams of sample was extracted in 20ml methanol after shaking of 15 min. Filtrate obtained was kept for drying and then the residual mass was treated with 1% H_2SO_4 (5ml.) for 2 times. After this, extraction was performed in 10ml. Chloroform using separating funnel. The chloroform layer which was organic by nature was rejected and the other aqueous layer was basified utilizing 30% NH_4OH of PH=9- 10. Again basified layer was extracted in 10ml chloroform and organic layer of chloroform which was in a lower position was collected in a flask and the step was repeated with fresh chloroform. Extracts was thus obtained was dried in vacuum for further use.

***Steroid Extraction*:** Steroids were extracted from bark of *T. arjuna* by well established method (15). The Fine powdered hundred gram of sample of plant bark was extracted in petroleum ether for two to four hours. Then it was filtered and residual mass was treated with 15% ethanolic HCl for four hours. Further it was extracted in ethyl acetate and washed with distilled water to neutralize the extract. To remove the moisture content of the neutral extract it was passed over Sodium sulphate and was dried in vacuum.

***Extraction in different polar and non polar solvents*:** Powdered bark of *T. arjuna* (20 g) was taken in three flasks and water, methanol and petroleum ether were used as solvent. The dried material and solvents were taken in a 1:10 ratio. Those were kept at soxhlet unit for complete one day. Obtained extracts were thus filtered and the filtrate was subjected to dry in vacuum to obtain extract. The residual extract that obtained was stored in a refrigerator at 4°C in sterile glass bottles.

***In vitro* alpha amylase inhibitory assay**

***Starch iodine assay*:** Screening of alpha amylase inhibitors were performed using Xiao et al method in test tubes with slight modifications based on the starch iodine test [16]. The assay mixture was about 120 ìl of 0.02M sodium phosphate buffer (pH 6.9), 1.5 ml of salivary alpha amylase and bark extracts at a concentration from 0.5-1.5 $mgml^{-1}$ (w/v) were incubated at 37°C for 10 min. After that, soluble 1% starch was added at each reaction mixture and incubated at 37°C for 15 min. Then 60 µl of 1 M HCl was added to the reaction mixture to stop the enzymatic reaction and immediately 300 µl of iodine reagent was added. If any change in colour was noted and at 620nm the absorbance was read. The plant extracts was not added to the control reaction showing 100% enzyme activity. Extract control was also included to check if any absorbance produced by bark extract. Thus different colour obtained indicates the presence of starch (dark-blue), absence of starch (yellow) and partially degraded starch (brownish) in the reaction mixture. If inhibitor was present in the extract of plant bark it inhibits the degradation of starch added to the enzyme assay mixture and form a dark-blue colour complex whereas no colour showed the absence of inhibitor.

***3, 5-dinitrosalicylic acid assay*:** The inhibition assay of bark extract of *T. arjuna* was performed using the chromogenic DNSA method (17). The assay mixture consists of 500 µl of 0.02 M sodium phosphate buffer (pH 6.9), 1ml of salivary alpha amylase and 400 ìl extracts at concentrations from 0.5-1.5 $mgml^{-1}$ were incubated at 37°C for 10 min. After pre-incubation, 580 µl of 1% starch solution was added at each tube and incubated at 37°C for 15 min. Using 1.0 ml DNSA reagent the reaction was terminated and tubes were placed in boiling water bath for 5 min. After this, cooled to room temperature and at 540nm the absorbances were measured. The control did not contain any bark extract represented 100% enzyme activity. Extract control was also a part to check if any absorbance produced by bark extract except for the enzyme.

Formula for calculation of the percent inhibition of alpha amylase:

% Relative enzyme activity = (enzyme activity of test/enzyme activity of control)×100.

Percent Inhibition of the α-amylase activity = (100"% Relative enzyme activity).

***Statistical analysis*:** Experiments were performed in triplicates for three different sets and ± standard error of the mean was used for calculation. Graph pad prism5 software was used for ANOVA, linear regression and statistical difference analysis. The IC_{50} values were calculated.

Results

Out of the solvent extracts methanol extract exhibited maximum inhibition of alpha amylase activity as compared to free flavonoid, bound flavonoid, alkaloid, steroid and other solvent extract extracts. The maximum activity of methanol extract may be due to presence of potential inhibitory compound in extract. Water, petroleum ether, alkaloid and steroid extracts showed negligible inhibitory activity with insignificant IC_{50} value.

***Extract with maximum inhibitory effect on the alpha amylase activity*:** Methanolic extracts (at a concentration 0.5-1.5 mg/ml) showed maximum α-amylase inhibitory activity from 46.93±0.03% to 48.26±0.03% with an IC_{50} value of 5.1642 mg/ml. At the same concentration free flavonoid extracts also showed good inhibitory activity i.e. 40.97±0.08% to 43.23±0.06 % with an IC_{50} value of 38.2825 mg/ml (Table-1).

***Extracts with insignificant inhibitory effects on the α-amylase activity*:** Water, petroleum ether, alkaloid and steroid extracts showed minimum inhibitory activity from 25.50±0.12% to 26.13±0.03%, 10.13±0.03% to 12.13±0.09%, 1.23±0.03 % to 2.63±0.13% and 1.70±0.01% to 2.37±0.06% respectively with an insignificant IC50 value (Table-2).

Discussion

Plants produce the secondary metabolites like flavonoids, tannins, alkaloid, and other serve

Table 1. Extract with maximum inhibitory effect

Sr. No.	Name of extract	Concentration Mg/ml	% inhibition	Regression Equation	IC_{50} value (mg/ml)
1	Methanol	0.5	46.93±0.03	Y=4.928+0.101X	5.1642
		1.0	47.63±0.03		
		1.5	48.26±0.03		
2	Free flavonoids	0.5	40.97±0.08	Y=4.799+0.127X	38.2825
		1.0	42.30±0.01		
		1.5	43.23±0.06		
3	Bound flavonoids	0.5	30.67±0.12	Y=4.53+0.168X	628.0584
		1.0	32.47±0.13		
		1.5	33.16±0.13		

Values are given as mean±SEM (n=3), ANOVA was used which show significant difference with respect to control (P<0.05).

Table 2. Extract with insignificant inhibitory effect

Sr No	Name of extract	Concentration Mg/ml	% inhibition	Regression	IC_{50} value(mg/ml)
1	Water	0.5	25.50±0.12	Y=4.342+0.056X	56231325190.391
		1.0	25.93±0.05		
		1.5	26.13±0.03		
2	Petroleum ether	0.5	10.13±0.03	Y=3.779+0.205X	90369.737
		1.0	11.03±0.06		
		1.5	12.13±0.09		
3	Alkaloids	0.5	1.23±0.03	Y=2.883+0.624X	2471.7241
		1.0	2.17±0.03		
		1.5	2.63±0.13		
4	Steroids	0.5	1.70±0.01	Y=2.883+0.624X	2471.7241
		1.0	2.06±0.08		
		1.5	2.37±0.06		

Values are given as mean±SEM (n=3), ANOVA was used which show significant difference with respect to control (P<0.05).

as a defense agent against pathogens and various other disorders. Diabetes is part of the chronic diseases that occur either when the level of insulin (a hormone) decreases or when the body is unable to use the insulin produced. In a recent WHO report, it is reported that more than 400 million people live with diabetes (18). Inhibition of alpha amylase by an agent is being considered to be effective in controlling diabetes. In present investigation bark extracts of *T. arjuna* were tested for their antidibetic activity. In one of the previous study the methanolic extracts of the leaves of

Terminalia arjuna, *T. bellerica*, and *T. chebula* were evaluated for hypoglycemic screening and an oral glucose tolerance test (OGTT) in normal rats and showed that *T. chebula* extract had a better hypoglycemic effect in normal and glucose induced hyperglycemic rats (19). In present study it was observed that methanolic extract of bark of showed significant % inhibition of alpha amylase at all tested concentrations with good IC_{50} value. All previous study was based on a test of one of the crude extract of *T arjuna* for their hypoglycemic effect and there is not any comparative study of all extract for their effect on inhibition of alpha amylase activity. Hence there is meager literature available to compare the results obtained in the present study. In this study, we compared IC_{50} value of α-amylase inhibitory activity of the crude extract of steroids, free flavonoids, bound flavonoids and alkaloids isolated from the bark of *T. arjuna* with polar and non polar solvent. Further studies are required to determine the mode of action of these plant extracts as alpha amylase enzyme inhibitors and to qualify the action of different constituents in the extract. The results of this study directs further researches to evaluate the therapeutic potentialities of methanolic, free flavonoids and bound flavonoids of bark of *T. arjuna* in the management of diabetes either alone or in a combinatorial therapy.

Conclusion

The present study showed that the methanolic extract of bark of *T. arjuna* showed maximum inhibition of alpha amylase activity with less IC_{50} value. Hence the extract may be useful as better therapeutic agent especially for the treatment of diabetes mellitus.

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Phytochemical and Hepatoprotective study of *Nymphaea nouchali* in Experimental Rats

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Abstract

The primary objective of present study was to explore the hepatoprotective activity of *Nymphaea nouchali* flower extract against carbon tetrachloride (CCl_4) induced liver necrosis. *Nymphaea nouchali* flowers were extracted with organic solvents like ethanol and n-hexane. These extracts were subjected to phytochemical studies. After the confirmation of flavonoids in the extracts, they were tested for *in-vivo* hepatoprotective activity. The study was carried out by taking female Wister rats in five groups, where each group consists of five rats. Biochemical and histological studies were carried out as a testimonial of hepatoprotective effect. Increased levels of biochemical tests like Albumin, direct bilirubin, indirect bilirubin, total bilirubin, serum glutamate-O-methyl transferase (SGOT), serum glutamate pyruvate transferase(SGPT) and alkaline phosphate (ALP) provide an evidence of liver necrosis in CCl4 induced group. Pre-treatment with *Nymphaea nouchali* ethanolic extract (NNEE) showed a significant recovery effect equally as that of the standard drug (silymarin) when compared with Nymphaea nouchali n-hexane extract (NNHE). The hepatoprotective effect was statistically analyzed by ANOVA and was found to be significant ($P<0.0001$).

Key words: *Nymphaea nouchali*, Flavonoid, Silymarin, Carbon tetrachloride, hepatoprotective

Introduction

Nymphaea nouchali is commonly called as water lily and it is an emerging aquatic plant found in ponds and lakes. It is a perennial plant belongs to the family of Nymphaeaceae and genus of Nymphaea (1). *Nymphaea nouchali* is the national flower of Bangladesh which consists of various secondary metabolites like alkaloids, tannins, sterols, saponins and flavonoids. It is a well known medicinal plant widely used in the Ayurveda and Siddha system of medicine for the treatment of liver disorders diabetes (2), inflammation (3), anti bacterial (4), urinary disorders, menstrual problems, as an aphrodisiac and as a bitter tonic.The liver is the principal organ for biotransformation. It is the largest gland which helps to metabolise the prodrugs and release the active constituent into the systemic circulation (5). It also helps to eliminate the drug after its pharmacological action. Certain drugs or chemicals in excess dose will act as toxins (6). These toxins after entering the liver will interact with hepatic cells and cause liver damage or injury. More than 900 drugs like non-steroidal anti-inflammatory drugs, glucocorticoids, hydrazine derivatives, etc., have been implicated in causing liver injury (7). Carbon tetrachloride (CCl_4) is a non-polar organic solvent which is widely used for hepatotoxicity studies in animal models. Recent studies have proved that the metabolites of CCl_4 induce acute and chronic tissue damage through bioactivation of phase I cytochrome P450 system. This bioactivation results out in the formation of peroxy trichloromethyl radicals ($\bullet OOCCl_3$) and trichloromethyl radicals ($\bullet CCl_3$) (8). These free radicals will then bind to the proteins, lipids and

other macromolecules. The macromolecules upon covalently bonding with the free radicals help to increase the concentration of lipo-peroxide and free peroxide radical that causes cell injury or necrosis. These free radicals will alter the cholesterol profile, damages the DNA by genetic mutations, chromosomal alteration and DNA strand breakage (9, 10). Silymarin is a flavonoid complex containing silybin, silydianin and silychrisin. It obtained from seeds of Silybum marianum (also called as milk thistle) (11). This species is an annual or biennial plant belongs to the family Asteraceae. Silymarin is an antioxidant hence widely used as the hepatoprotective agent. It scavenges for free radicals and helps to regenerate the hepatocells damaged by alcohol or drugs (12, 13). The aim of this present research study was to screen the phytochemical constituents present in *Nymphaea nouchali* flower extacts and also to investigate the hepatoprotective effect of *Nymphaea nouchali* flower against carbon tetrachloride induced liver damage in albino rats.

Materials and Methods

Drugs and reagents: Silymarin was acquired from Sigma-Aldrich, Bangalore, India. Carbon tetrachloride (CCl_4) was obtained from Thermo Fisher Scientific India Pvt. Ltd, Mumbai, India. Formalin was obtained from Sigma-Aldrich, Bangalore, India.

Plant material: Nymphaea nouchali belongs to the family Nymphaeaceae. The flowers were collected from the field trips nearby ponds and lakes in Guntur district, Andhra Pradesh. The plant material was authenticated by Dr. K. Ammani, Department of Biotany and Microbiology, Acharya Nagarjuna University. After the collection, the flowers were rinsed with distilled water and then air-dried under the shade. The dried flowers were then powered and stored in air-tight container.

Extraction: The air dried flower was powdered coarsely and 250gms of powder will be weighed and soaked in 1 litre of n-hexane by maceration. The mixture will be kept for three days in a tightly sealed vessel at room temperature and stirred several times daily with a glass rod. This mixture will be filtered through Whatman No-1 filter paper[14]. The extract will be subjected to rotavapor evaporation to remove the solvent. The same procedure will be used for the ethanolic extraction. The course powder which used in maceration is again re-utilised for soxhlet extraction. The extracts obtained will be kept in a desiccator containing calcium chloride[15]. These extracts will be stored at -4ºC until use and dissolved in normal saline on the day of the experiments for the purpose of phytochemical screening and evaluating pharmacological activity.

Phytochemical study of Flower extracts: Phytochemical screening was carried out to detect the presence of various active constituents using qualitative tests like molisch test, fehlings test, benedict's test for identification of carbohydrates (16), biuret test and ninhydrin test for identification of proteins (16), test for dragendorff's, hager's, wagner's and mayer's was performed for identification of alkaloids (17), bromine water test and schoenteten's test (Borax test) for identification of glycosides. Steroids were identified by salkwoski and liebermann-Burchard test, flavonoids (18) were identified by ammonia test and zinc chloride test, tannins were identified by ferric chloride test, lead acetate test and also test for fixed oils, saponnins and fatty acids (18) were performed using standard operating procedures.

Experimental animals: Wister rats of either sex were commonly employed for hepatotoxicity studies. In the present study, female rats of 150 to 250 gm weight were procured Mahaveer Enterprises, Hyderabad and then housed/ maintained at 23-25ºC under the light and dark 12 hr cycle with 60-80% relative humidity (as per CPCSEA guidelines). The rats were fed with Amrut® and water. All female Wister rats were placed in laboratory one week prior to experimentation in order to acclimatize them to the laboratory conditions. The protocol for experimentation procedure was approved by Institutional Animal Ethics Committee (IAEC) of Chalapathi Institute of Pharmaceutical Sciences, Guntur, Andhra Pradesh (Approval No: 17/IAEC/ CIPS/2016-17; data 05/04/2016).

Experimental groups: The hepatoprotective activity of *Nymphaea nouchali* ethanolic extract (NNEE) and *Nymphaea nouchali* n-hexane extract (NNHE) were evaluated by comparing with the standard silymarin through *in-vivo* hepatotoxic studies on female Wister ratsTotal of five groups was taken, for the study of hepatoprotective activity in *Nymphaea nouchali* flower, where each group consists of five female Wister rats.

Group-I (Normal control): Rats were treated with normal saline orally for 14 days.

Group-II (Disease control): Rats were treated with normal saline orally for 14 days and on 14th day they were administered with CCl_4 (3ml/Kg body weight)

Group-III (Standard): Rats were treated with silymarin (50mg/Kg body weight) orally for 14 days, and on the 14th day silymarin was administered 60 min prior the administration of CCl_4.

Group-IV (n-hexane extract): Rats were treated with NNHE (200mg/Kg body weight) orally for 14 days, and on 14th day NNHE was administered 60 min prior the administration of CCl_4.

Group-V (Ethanolic extract): Rats were treated with NNEE (200mg/Kg body weight) orally for 14 days and on 14th day NNEE was administered 60 min prior the administration of CCl_4.

Parameters estimated: After the course of treatment, the animals will be placed in laboratory conditions for one hour and then anatomised under ether anaesthesia. From jugular vein blood samples were collected for various biochemical estimations.

***Biochemical parameters*:** Albumin (g/dl), direct bilirubin (mg/dl), indirect bilirubin (mg/dl), total bilirubin (mg/dl), serum glutamic oxaloacetic transaminase (SGOT, U/lit), serum glutamic pyruvate transaminase (SGPT, U/lit), alkaline phosphatase test (ALP, U/lit)

Statistical analysis: Results of biochemical estimations were reported as a mean ± standard error of means (S.E.M.) for the determination of significant intergroup difference (P-value <0.001) and a one-way analysis of variance (ANOVA) followed by Tukey's multiple range test using graph pad prism version 6.0.

Results and Discussion

Various qualitative chemical tests have been performed for the phytochemical study of *Nymphaea nouchali* flower extracts and results were delineated in table-1. In the present *in-vivo* study for hepatotoxicity, CCl_4 treated group (Group-II) showed a significant increase in all blood biochemical parameters except direct bilirubin ($P<0.0001$) test when compared with normal control group (Group-I) Silymarin treated group (Group-III), gives an increased levels of indirect bilirubin and total bilirubin test, decreased levels of albumin ($P<0.0001$), SGPT ($P<0.01$), SGOT ($P<0.01$) and ALP ($P<0.01$) test, when compared with diseased control group (GROUP-II) (5,6). It also showed an increased level of albumin, indirect bilirubin ($P<0.0001$), total bilirubin ($P<0.0001$), SGPT and ALP test, whereas it showed a decreased levels of direct bilirubin and SGOT test when compared with normal control group (Group-I) (9).

Nymphaea nouchali n-hexane extract pre-treated group (Group-IV), showed a significantly increased level of direct bilirubin test, SGOT, ALP test and a significant decreased levels of indirect bilirubin, total bilirubin and albumin test, when compared with silymarin treated group (Group-III) (11, 13). NNHE pre-treated group, showed a decreased levels in all biochemical tests except in direct bilirubin ($P<0.0001$) and ALP, when compared with diseased control group (Group-II). It also showed a significant decrease in biochemical tests except for SGOT ($P<0.01$), SGPT ($P<0.01$) and ALP ($P<0.01$), when compared with normal control group (Group-I)*Nymphaea nouchali* ethanolic extract pre-treated group (Group-V), showed a significantly increased level of all biochemical tests when compared with

Nymphaea nouchali n-hexane extract pre-treated group (Group-IV). NNEE pre-treated group, showed increased levels in biochemical tests except for albumin ($P<0.0001$), indirect bilirubin ($P<0.0001$) and SGPT ($P<0.01$), when compared with silymarin treated group (Group-III). It also showed a significantly decreased level in biochemical tests except for direct bilirubin ($P<0.0001$), total bilirubin ($P<0.0001$) and ALP ($P<0.01$), when compared with diseased control group (Group-II). NNEE pre-treated group, showed an increased level except for albumin and direct bilirubin test when compared with normal control group (Group-I).

In Histopathological studies, CCl_4 induced hepatotoxicity group showed significant damage in liver tissue, when compared with control group (Fig. 4). CCl_4 pre-treated group reported a periportal inflammation and necrosis when compared to control group which maintained unchanged liver tissue architecture. Silymarin pre-treatment group showed central vein dilatation when compared with CCl_4 pre-treated group. The histological slides of NNHE pre-treated group showed central vein dilatation of liver tissue whereas NNEE pre-treated group showed a significant recovery even after the administration of CCl_4 which were depicted in fig. 4. The Hepatoprotective activity of Nymphaea nouchali plant is due to the presence of flavonoids and the existing research work conforms that this plant is well known for its anti-bacterial, anti-inflammatory and anti-diabetic activity. The present study was a novel research from which we can conform that Nymphaea nouchali flower can be used even for hepatoprotective activity.

Conclusion

From the results obtained by Phytochemical study, both the extracts contain flavonoids which have a potent hepatoprotective activity. When CCl_4 is administered, the hepatoprotective activity of *Nymphaea nouchali* ethanolic extract showed significant recovery of tissue necrosis when compared with Nymphaea *nouchali* n-hexane extract.

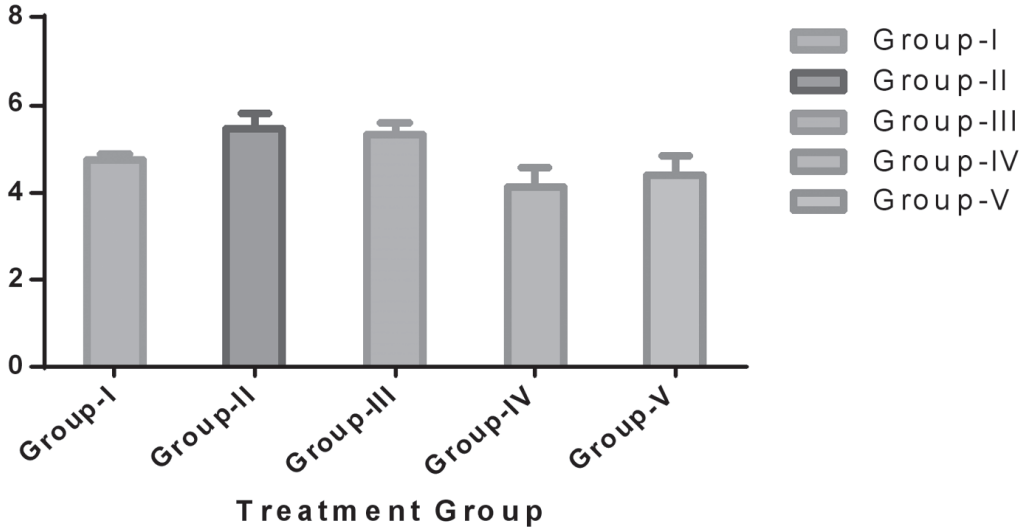

Fig.1. Effect of *Nymphaea nouchali* flower extracts on Albumin in various treatment groups

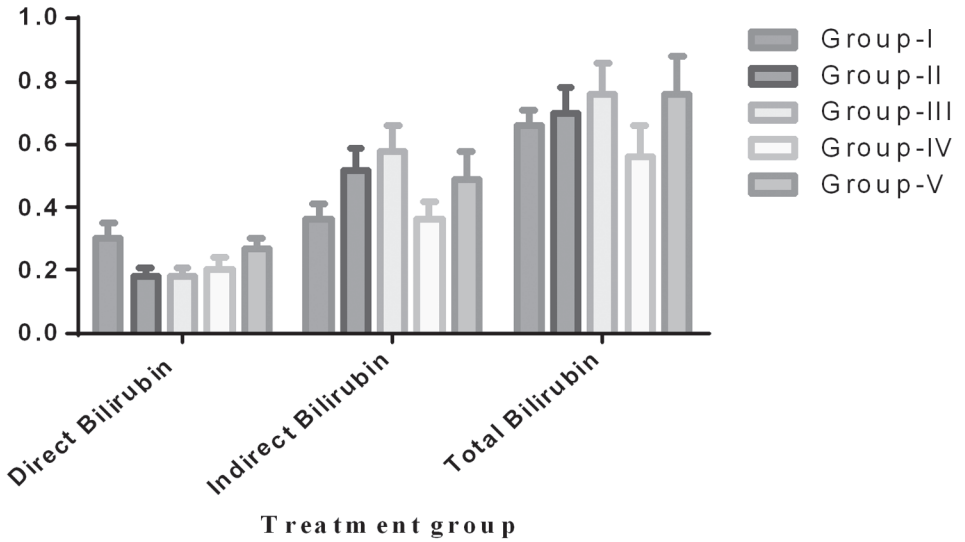

Fig. 2. Effect of *Nymphaea nouchali* flower extracts on direct, indirect and total bilirubin in various treatment groups

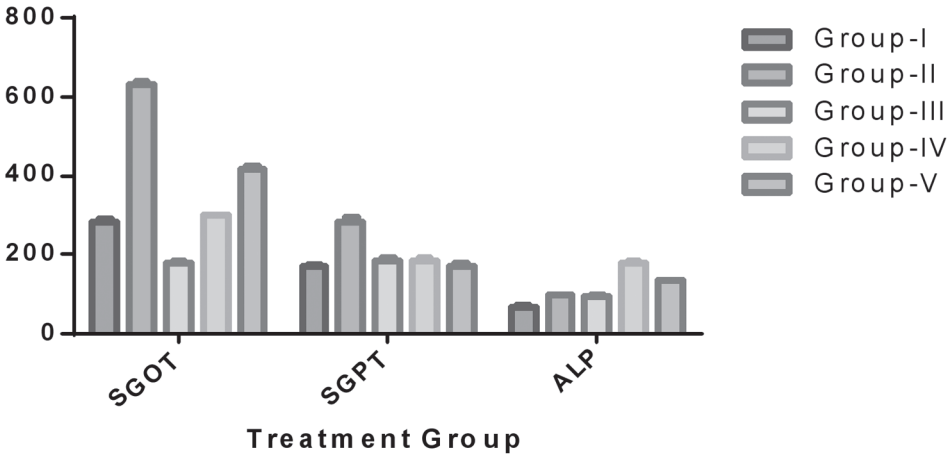

Fig. 3. Effect of *Nymphaea nouchali* flower extracts on SGOT, SGPT and ALP in various treatment groups

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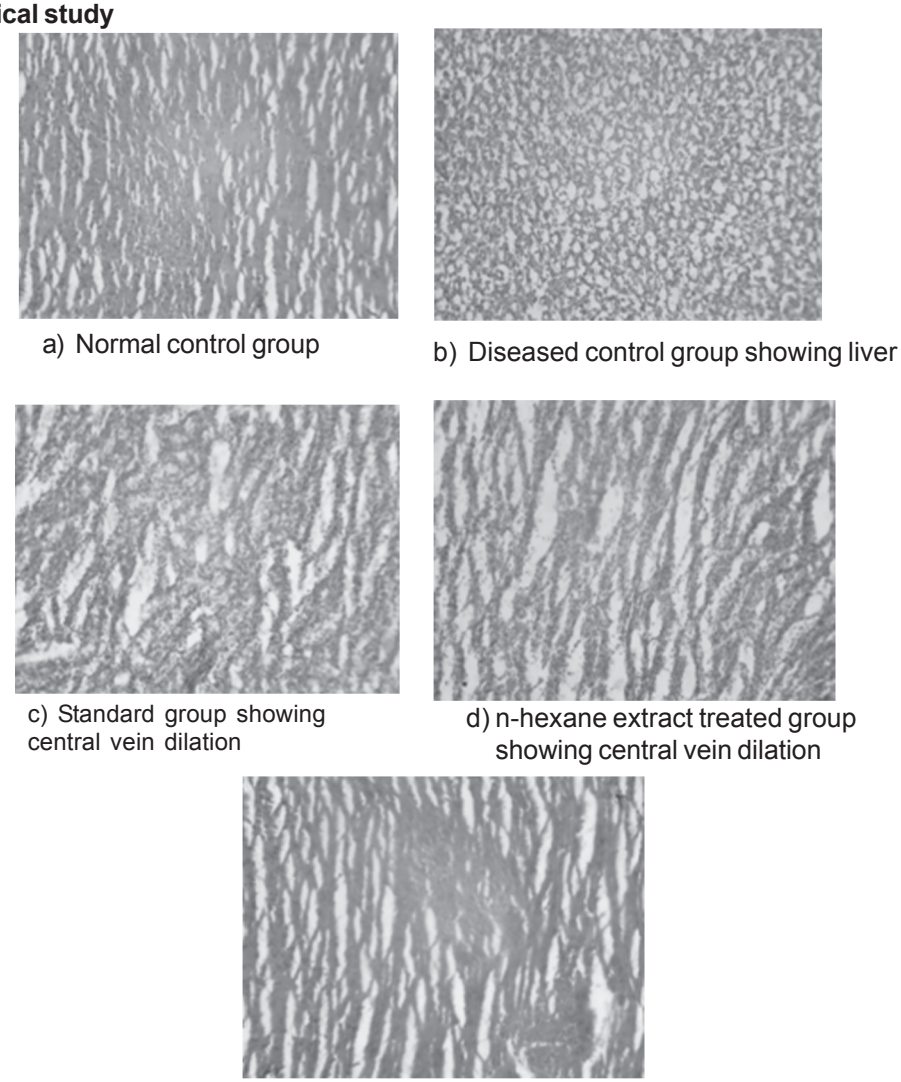
Histopathological study



a) Normal control group

b) Diseased control group showing liver

c) Standard group showing central vein dilation

d) n-hexane extract treated group showing central vein dilation

e) Ethanolic extract treated group showing Significant recovery when compared to diseased control group

Fig 4. Histopathology study of isolated liver of various treatment groups showing haematoxylin and eosin stained cells

Table 1. Phytochemical constituents of *Nymphaea nouchali* flower extracts

S.No	Phytoconstituent	*Nymphaea nouchali* ethanolic extract	*Nymphaea nouchali* n-hexane extract
01	**Carbohydrates**		
	Molisch test	—	—
	Fehlings test	—	—
	Benedict's test	—	—
02	**Proteins**		
	Biuret test	—	—
	Ninhydrin test	—	—
03	**Alkaloids**		
	Dragendorff's test	++	—
	Hager's test	++	—
	Wagner's test	++	—
	Mayer's test	++	—
04	**Glycosides**		
	Bromine water test	—	—
	Schoenteten's test (Borax test)	—	—
05	**Steroids**		
	Salkwoski test:	++	++
	Liebermann-Burchard test	++	++
06	**Saponins**	—	—
07	**Flavonoids**		
	Ammonia test	++	++
	Zinc chloride test	++	++
08	**Tannins**		
	Ferric chloride test	—	—
	Lead acetate test	—	—
09	**Fixed oils**	—	—
10	**Fatty acids**	++	—

Table 2. Biochemical Parameters of various treatment Groups

S.NO	Parameter	Group-I	Group-II	Group-III	Group-IV	Group-V
01	Albumin (g/dl)	4.75±0.11	5.44 ±0.38	5.32±0.27	4.14±0.41	4.38±0.46
02	Direct Bilirubin(mg/dl)	0.3±0.05	0.18±0.03	0.18±0.03	0.2±0.04	0.27±0.03
03	IndirectBilirubin(mg/dl)	0.36±0.05	0.52±0.07	0.58±0.08	0.34±0.06	0.50±0.09
04	Total Bilirubin(mg/dl)	0.66±0.05	0.7±0.08	0.76±0.1	0.54±0.1	0.77±0.12
05	SGOT (IU/L)	284.4±7.5	632±6.3	180.6±3.5	298.8±3.7	416.8±6.8
06	SGPT (IU/L)	170.6±4.9	284.2±10.1	183.8±9.5	183.8±10.9	172.8±6.6
07	ALP (IU/L)	69.8±2.2	97.8±1.7	95.6±2	178.2±4.9	133±4

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Radiological: Pre-clinical Comparative study of Microbial derived Surfactants with Survanta for Treatment of Respiratory Distress Syndrome (RDS)

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Abstract

Present study was to do comparative study of microbial produced surfactant with FDA approved drug survanta by using Near IR and Far IR spectroscopy this is useful for pre clinical test for RDS treatment. More recently IR spectroscopy comes as novel diagnostic tests to assess fetal lung maturity. Two spectral diagnostic windows for deep tissue imaging in Near IR spectrum (300-1100nm) and for Far IR spectrum (1200nm-2200nm) were used for comparable study of drug survanta with microbial produced Surfactin and Lipopeptide (Biosurfactant) from *B.subtilis* MTCC2423 and *C.tropicalis* MTCC230 respectively. Near IR spectrum (300-1100nm) shows the absorbance peak for surfactin and lipopeptide (Biosurfactant) was fall in same wavelength as survanta shows i.e, 396nm and 967nm. Far IR spectrum (1200nm-2200nm) shows that the least absorbance frequency was obtained at 1249nm for all the three surfactants. Surfactin shows minimum absorbance unit in highest dilution then followed by lipopeptide (Biosurfactant) supernatant then survanta i.e., 0.17, 0.24 and 0.42AU respectively. Surfactin and Lipopeptide (Biosurfactant) produced from *B.subtilis* MTCC2423 and *C.tropicalis* MTCC230 respectively, can be considered for RDS treatment as it clear the primarily study of radiological property of drug by comparable study with FDA approved drug survanta.

Keywords: Respiratory distress syndrome, Surfactin, Lipopeptide, Near IR, Far IR.

Introduction

Lungs are lined by a surface active material called the pulmonary surfactant which prevents alveolar collapse and maintains alveolar stability. Pulmonary surfactant (PS) is a complex mixture of lipids and proteins that coats the interior surface of the vertebrate lung as a film. It consists of about 90% lipids (mainly phospholipids) and 8–10% protein. The main component of native Lung surfactant (LS) is dipalmitoyl-phosphatidylcholine (DPPC) an amphiphilic protein that were recently shown to play an important role in the surface activity of LS (1,2). Lack of deficiency or inactivation of PS causes severe respiratory disorders that can be lethal such as breathing problems in newborn babies, pulmonary haemorrhage, pulmonary oedema, and Pneumonia, respiratory distress syndrome (RDS)- a most common causes of death in premature infants (3,4).

However, in general probability for RDS is more prominent in premature infant because lungs are under developed, so the lung surfactant production is less. In Most cases of RDS babies were born before 37 weeks. If a premature baby is lacking surfactant, artificial surfactant may be given for normal functioning (5-7).

Amount of surfactant present in fetal lungs can be estimated by measuring the amount of surfactants in amniotic fluid (8). Treatment of neonates immediately after birth with exogenous surfactant delivered endotracheally can be

effective if early delivery cannot be prevented (9). In the medical research, imaging and diagnosis via a infra-red (IR) imaging has been increased due penetration in deep tissue imaging (10). Diagnosis of RDS noninvasively, primary test is chest radiography which is used as one of the most usual and accessible diagnostic tools in respiratory distress syndrome, which has shown promising results regarding the early diagnosis of respiratory distress (11). IR spectroscopy is one of a standard clinical analyses test of amniotic fluid assays to assess fetal lung maturity (12).

In a clinical study and radiographic diagnosis matched upto 95% of cases (13)], In another study the specificity and sensitivity of the radiographic test was 82.5% and 35.8% respectively (14) and in the same study author concluded that diagnostic value of RDS based on radiologist opinion were about one-third of all cases and has low sensitivity. Although we have many options/ methods to diagnose RDS but still there is not a single non invasive method which can be 100% reliable.

In 1990, the United States Food and Drug Administration (FDA) released colfosceril palmitate for use in preterm infants with RDS. Shortly thereafter, other types of surfactants became available: Exosurf® (synthetic surfactant) and Survanta® (modified natural surfactant). Also domestically available are Curosurf® (natural surfactant) and Infrasurf® (natural surfactant) (15). Animal derived surfactants are mostly used as drugs for the treatment of respiratory distress syndrome (RDS) in neonates. They also have several disadvantages such as they carry the potential risk of transmission of infectious agent as well as exposure to foreign proteins, pro-inflammatory mediators and variability in a native surfactant proteins from batch to batch (16). In this study we have taken survanta as standard for the comparative analysis because this is most common one, have highest DPPC composition and cost-effective.

The objective of present study was to do comparative study of microbial produced surfactant with FDA approved drug survanta by using Near IR and Far IR spectroscopy this is useful for pre clinical test for RDS treatment. Lim et al. (17) identified two spectral diagnostic windows for deep tissue imaging in Near IR region of the spectrum (700–900 nm) and for Far IR spectrum 1,200– 1,600-nm, these two IR spectrum region used as “diagnostic window” of tissue imaging, where the absorption coefficients of body fluids are at their minimum (12).

In this study we analysed the Near IR and Far IR spectrums of three different surfactants (Survanta, surfactin, lipopeptide). Spectrums were obtained from different concentrations of surfactant were compared and analyzed. The results showed that there is a specific set of frequencies for all three surfactants and these set of frequencies can be used to detect the presence of surfactant. Survanta (FDA approved drug used for RDS treatment) was used as a standard for the comparative radiological study of surfactin produced from *B.subtilis* MTCC 2423 and lipopeptide (Biosurfactant) produced from *C.tropicalis* MTCC230. This study reveals that microbial surfactant may be considered for treatment of RDS in an infant.

Materials and Methods

We analysed the spectrum of Survanta (manufactured by: M/s AbbVie Inc. 1401, Sheridan Road, North Chicago, II, 60064, USA) , Surfactin (*B.subtilis* MTCC 2423) and Lipopeptide (*C.tropicalis* MTCC230) were produced in Protein Engineering Laboratory, School of Biochemical Engineering, IIT (BHU), Varanasi (18,19).

A spectroscopic measurement was performed by using Aventes Spectrometer system which consists of:

1. Light source- AvaLight-HAL Tungsten Halogen Light Source, The AvaLight-HAL is a compact stabilized halogen fan-cooled light source that can be used for the visible and the near infrared range. Its wavelength range is 360-2000 nm.
2. Detectors: AvaSpec-2048 Fiber Optic Spectrometer, it is a 2048 pixel CCD

Detector Array. Usable wavelength range is 300-1100 nm. AvaSpec-NIR256-2.2TEC NIR Line Near-Infrared Fibre-optic Spectrometer, its wavelength range is 1000-2200 nm.

3. Cuvette sample holder
4. Fiber optic cables
5. Cuvette
6. Micropipette
7. Avasoft 8(software installed in a personal computer) was used to process data received from above detectors.

Sample preparation: Samples of different concentration of each surfactant (survanta, surfactin and lipopeptide) were prepared. Survanta was obtained from pharma market of varanasi, india (manufactured by: M/s AbbVie Inc. 1401, Sheridan Road, North Chicago, II, 60064, USA), Surfactin was produced from *B.subtilis* MTCC 2423 and lipopeptide (Biosurfactant) was produced from an adaptive strain of *C.tropicalis* MTCC230 (18,19). There supernatants were used as a pure sample for Near IR and Far IR spectrum studies.

***Comparative Study*:** Radiological Comparative studies of survanta, surfactin and lipopeptide (Biosurfactant) were performed by using Near IR spectrum of wavelength range is 300-1100 nm. Survanta act as a standard for the comparative study, further comparative studies were performed by using Far IR spectrum of wavelength range is 1000-2200 nm.

Concentration Variation: Radiological study of survanta, surfactin and lipopeptide (Biosurfactant) was performed at different concentration maintaining the final volume 2ml via a different dilution rate with distilled water.

Fermented broth of Supernatant containing surfactin and lipopeptide (Biosurfactant) used as pure sample then gradually increases the dilution with distilled water at dilution rate i.e., 50%, 62.5%, 75%, 87.5% respectively. Pure survanta is highly viscous so initial dilution rate was started from 0.20ml survanta + 1.80ml distilled water, then gradually increases the dilution rate as same in case of surfactin and lipopeptide. All samples were measured by using VIS-NIR spectrum of wavelength range is 300-1100 nm and Far IR spectrum of wavelength range is 1000-2200 nm.

Results

Comparative Study: Comparative Studies of surfactin and lipopeptide (Biosurfactant) with the survanta were performed by using Near IR spectrum of wavelength range is 300-1100 nm as shown in Figure 1, result shows that the absorbance peak frequencies for surfactin, lipopeptide (Biosurfactant) and survanta were 396nm and 967nm, So the absorbance peak for surfactin and lipopeptide (Biosurfactant) was fall in same wavelength as survanta shows, Absorbance units (AU) were different for each surfactant due to concentration difference. Similarly for Far IR spectrum, wavelength range 1200nm-2200nm used for comparative study of surfactin and lipopeptide (Biosurfactant) with the survanta, Figure 2 shows that the least absorbance frequency was obtained at 1249nm for all the three surfactants. These results reveal that microbial produced surfactin from *B.subtilis* MTCC2423 and lipopeptide (Biosurfactant) from an adaptive strain of *C.tropicalis* MTCC230 showing the same Spectroscopic properties for Near IR and Far IR as shown by drug survanta. This result is very much helpful to pass preclinical trials for the development of drug used in RDS (respiratory distress syndrome). Surfactin and lipopeptide (Biosurfactant) may considered as drug for the treatment of respiratory distress syndrome (RDS) in premature infants, as it clears the primarily preclinical test i.e., radiological study.

Further surfactin and lipopeptide (Biosurfactant) comparative studies with survanta were done on account of varying the concentrations, absorbance peak of each surfactant shows the concentration and presence of that surfactant so it's important to measure the absorbance peak by varying the concentrations.

Concentration Variation study: Results of Comparative study reveal that Surfactin and Lipopeptide (Biosurfactant) showing the same absorbance spectrum of Near IR and Far IR as

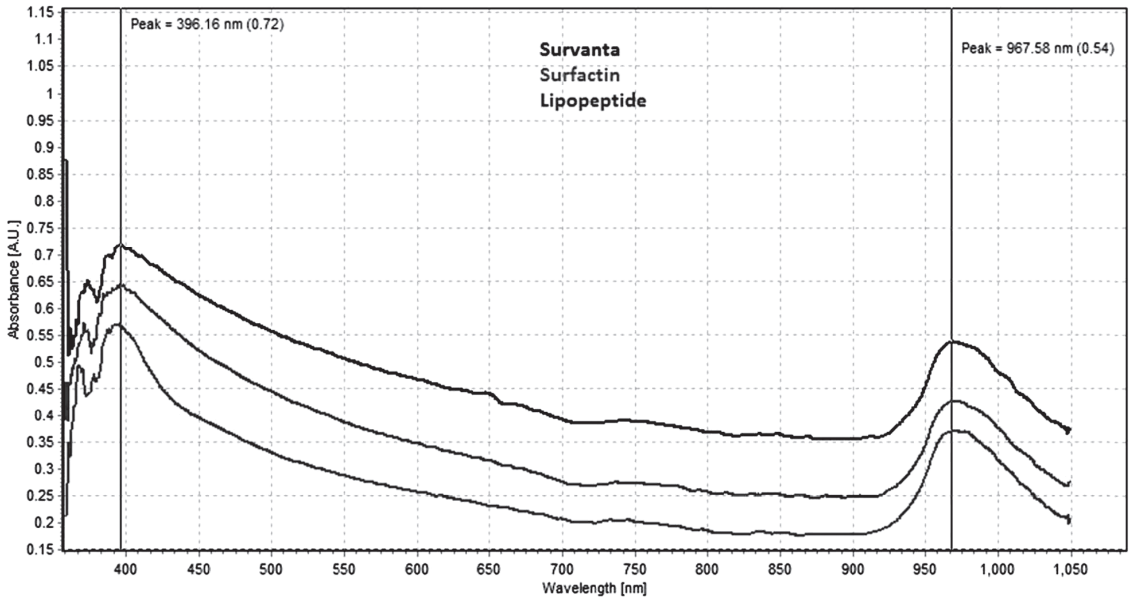

Fig. 1. Near IR spectrum of surfactin and lipopeptide (Biosurfactant) with the survanta.

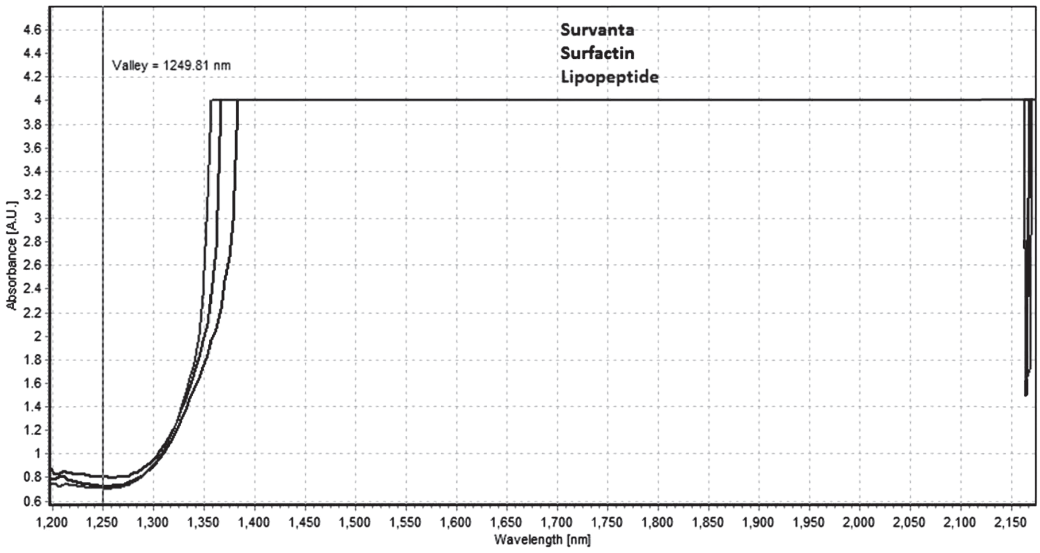

Fig. 2. Far IR spectrum of surfactin and lipopeptide (Biosurfactant) with the survanta

shown by survanta then their concentration variation studies were performed. Lipopeptide (Biosurfactant) produced from an adaptive strain *C.tropicalis,* was serially adapted from low to high concentrations of hydrocarbons (petroleum waste) (18,19). Supernatant of fermented broth of *B.subtilis* MTCC 2423 *and C.tropicalis* MTCC 230 containing surfactin and Lipopeptide (Biosurfactant) respectively, These Supernatants were used for analysing Near IR spectrometry and Far IR spectrometry with different dilutions.

Initially 2ml of supernatant was used for Near IR spectrum (range 300nm-1100nm), Supernatant containing Lipopeptide (Biosurfactant) showing absorbance 1.35 AU at 396nm (Figure 3) and Supernatant containing Surfactin show absorbance 0.59 AU at 369nm as (Figure 4), Absorbance Unit (AU) of 2ml supernatant containing the Lipopeptide (Biosurfactant) is nearly equal to pure drug survanta when diluted as 0.20ml of survanta + 1.80ml of distilled water at 369nm as shown in Figure 5.

To estimate the lowest absorbance unit for analysing the sensitivity and efficacy of Near IR and Far IR spectrum for microbial surfactants, dilution was increases gradually for lipopeptide (Biosurfactant) and surfactin containing supernatant, and also for survanta with the same dilution rate respectively.

Surfactin shows minimum absorbance unit in highest dilution then followed by lipopeptide (Biosurfactant) supernatant then survanta i.e., 0.17, 0.24 and 0.42AU respectively. Further Far IR spectrometry studies were also performed for each surfactants (Lipopeptide, surfactin and survanta) for different dilution rate as similar in case of Near IR, all the surfactants shows least absorbance at 1250nm for Far infrared spectrum range 1200nm-2200nm, this absorbance is showing very small change with change in concentration of Lipopeptide, surfactin and survanta as shown in Figure 6, 7, and 8 respectively.

Discussion

Near IR and Far IR spectrum are the two diagnostic windows considering how light interacts with living tissues, penetration of these IR into the living tissues can helps to diagnose that whether the lung contains normal or sufficient surfactants (20), this is one of most useful method for the determination of lung surfactant in neonates, as it can meet the standards of accuracy that are required for a specialized test such as amniotic fluid assays to assess fetal lung maturity. IR spectroscopic analysis having number of practical advantages like, No reagents are required, there is generally no need to dilute very concentrated specimens, very little sample is required (12).

Comparative Studies of surfactin and lipopeptide (Biosurfactant) with the survanta reveal that microbial produced surfactin from *B.subtilis* MTCC2423 and lipopeptide (Biosurfactant) from an adaptive strain of *C.tropicalis* MTCC230 showing the same Spectroscopic properties for Near IR and Far IR as shown by drug survanta. This result may consider as to cleared preclinical trials for the development of drug used in RDS (respiratory distress syndrome). So, Surfactin and lipopeptide (Biosurfactant) produced from *B.subtilis* MTCC 2423 *and* an adaptive strain of *C.tropicalis* MTCC 230 respectively, may considered as drug for the treatment of respiratory distress syndrome (RDS) in premature infants, as it clears the primarily preclinical test i.e., radiological study.

Further, surfactin and lipopeptide (Biosurfactant) comparative studies with survanta were done on account of varying the concentrations, absorbance peak of each surfactant shows the concentration and presence of that surfactant so it's important to measure the absorbance peak by varying the concentrations. This study was helpful to establish the sensitivity of NIR and Far IR absorbance spectrum with these surfactants, Different dilution rate shows different absorbance unit, as dilution rate increases absorbance unit decreases respectively.

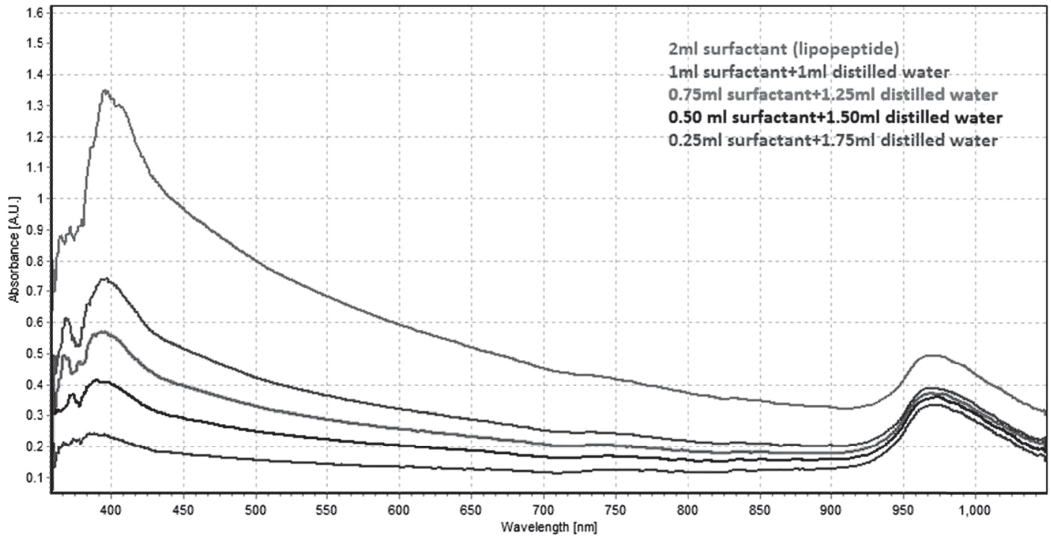

Fig. 3. Near IR spectrum of lipopeptide (Biosurfactant) at different dilutions.

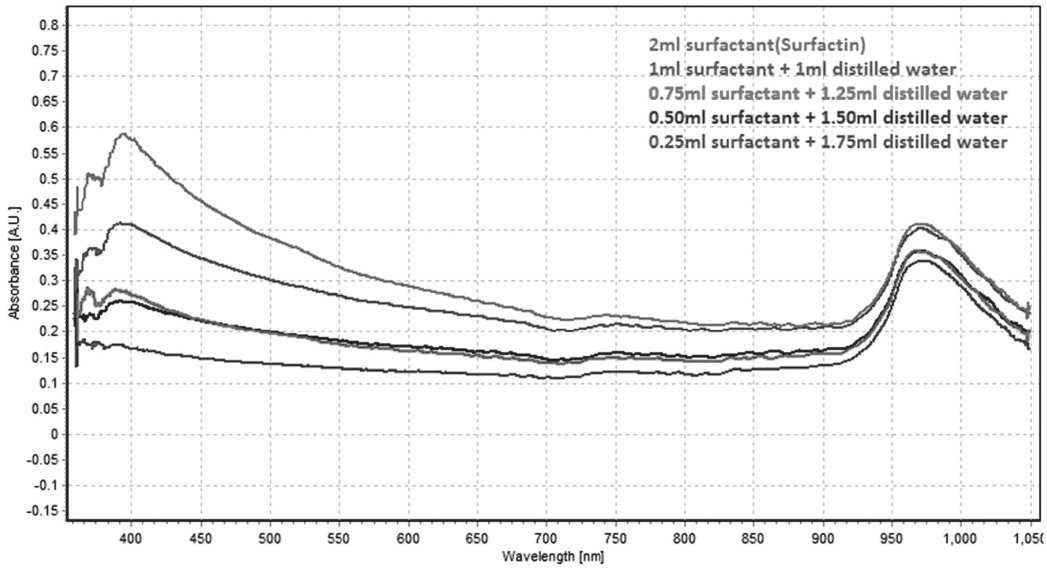

Fig. 4. Near IR spectrum of Surfactin at different dilutions.

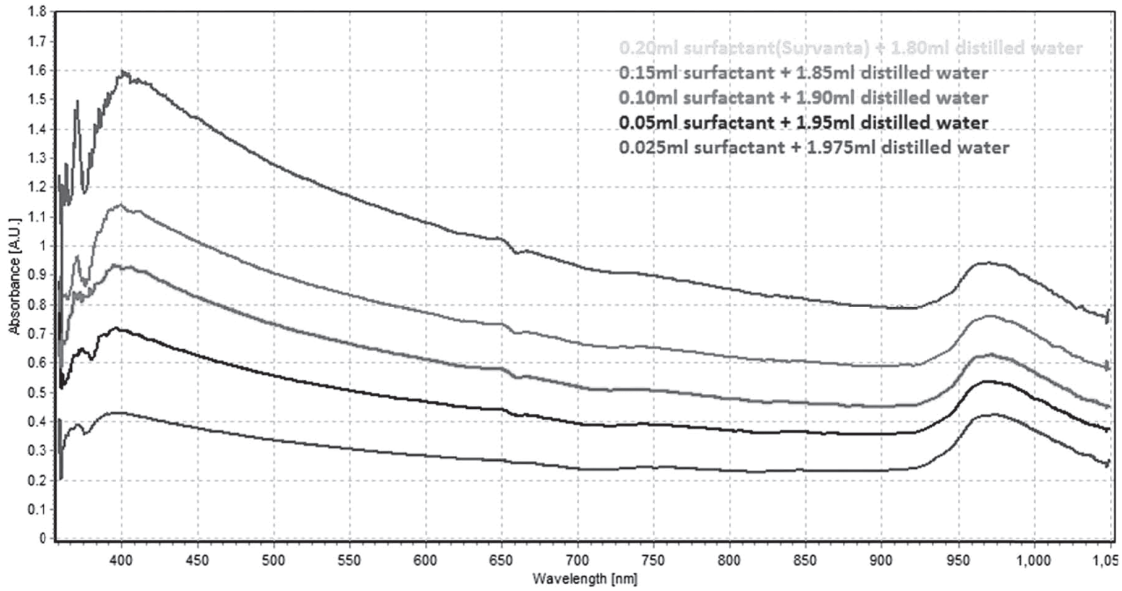

Fig. 5. Near IR spectrum of Survanta at different dilutions.

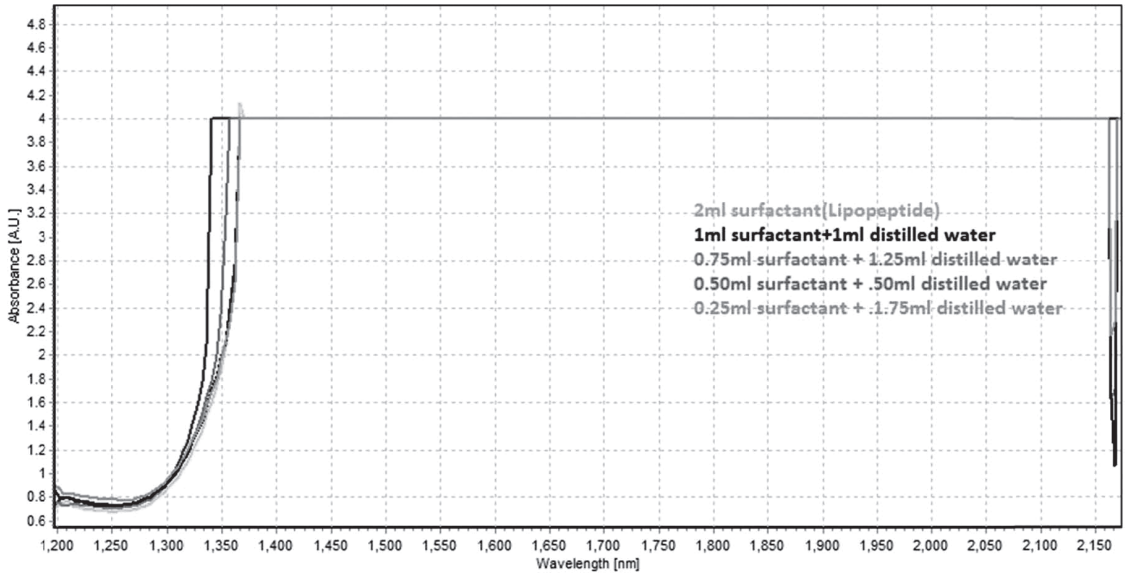

Fig. 6. Far IR spectrum of lipopeptide (Biosurfactant) at different dilutions.

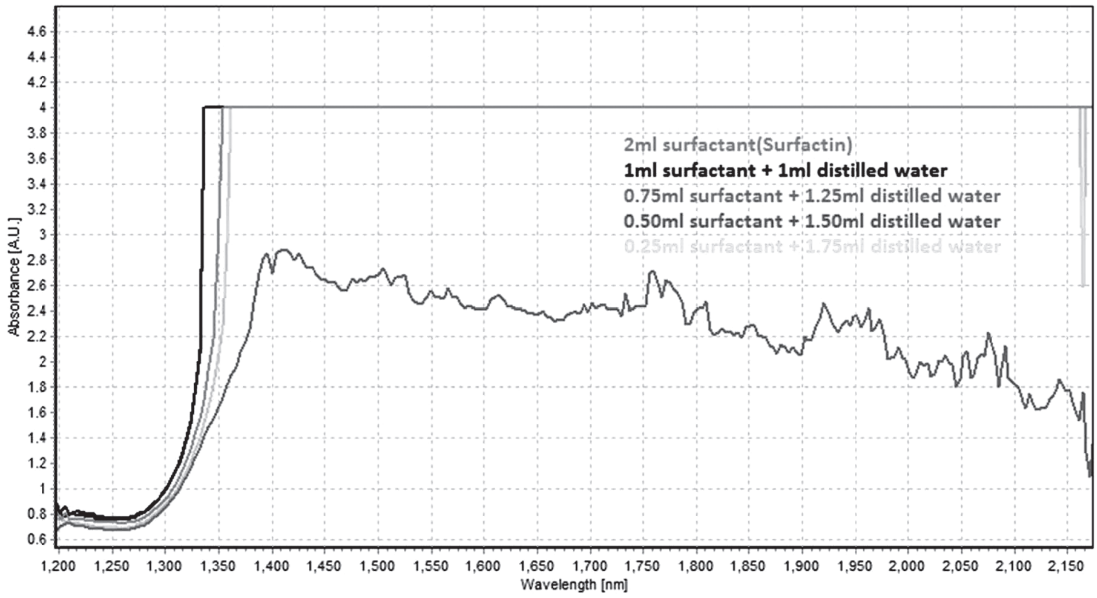

Fig. 7. Near IR spectrum of Surfactin at different dilutions.

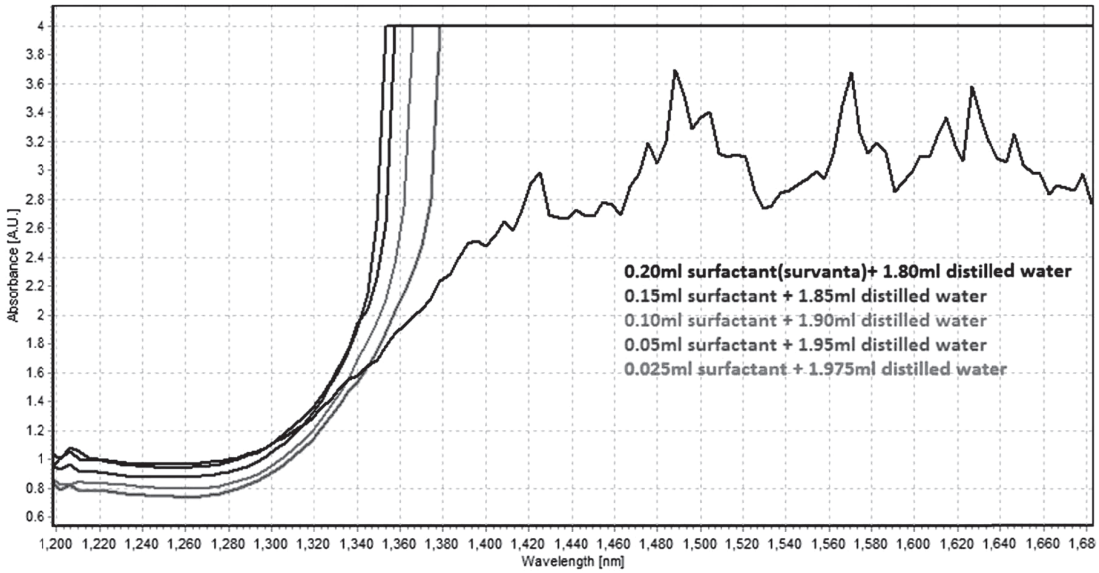

Fig. 8. Near IR spectrum of Survanta at different dilutions.

Results signifies that adaptive strain *C.tropicalis* MTCC 230 producing high amount of lipopeptide (Biosurfactant) then known strain of *B. subtilis* MTCC 2423 producing surfactin and the absorbance unit (AU) of Lipopeptide (Biosurfactant) is nearly equal to pure drug survanta at 369nm as shown in Fig. 5.

Absorbance of IR at particular wavelength shows the presence and concentration of surfactant inside the lungs, microbial synthesized surfactants i.e., surfactin and lipopeptide (Biosurfactant) showing the absorbance at 396nm and 960nm for Near IR and least absorbance is shown at 1250nm for Far IR spectrum is as same as survanta.

Conclusion

This study reveals that microbial surfactants can be considered as drug for the treatment of respiratory distress syndrome (RDS) in neonates, as these surfactants are eco friendly and non hazardous for body many of microbial synthesized surfactants are used for many diseases as a drug (21).

Surfactin and Lipopeptide (Biosurfactant) produced from *B.subtilis* MTCC2423 and *C.tropicalis* MTCC230 respectively, can be considered for RDS treatment as it clear the primarily study of radiological property of drug by comparable study with FDA approved drug survanta, showing the same IR pre clinical spectrometric results. Animal derived surfactants and chemically synthesized surfactants used for RDS treatment having some limitations like limited scale of production when animally derived and may hazardous when chemically synthesized over these limitations microbial synthesised surfactants having some advantages, it can be produced in large scale and found to be non-hazardous for human body. This work explore the potential use of microbial synthesized lipopeptide for the treatment of RDS as it passes the pre clinical trial of radiological study and so this information and data open the path for clinical and invivo study of microbial derived surfactant for RDS treatment in neonates.

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Chemical Characterization and Antidermatophytic Activity of *Thuja orientalis* Collected from Jaipur District, Rajasthan

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Abstract

Thuja orientalis commonly known as white cedar was collected from Rajasthan university campus, Jaipur District, Rajasthan, India. GC/GC-MS analysis of *T. orientalis* essential oil showed the presence of 38 compound for 100% of total oil. Delta-3-carene (30.37%) was the main component of thuja oil followed by alpha -pinene (13.11%), limonene (10.07%), alpha- terpinene (8.44%), alpha -terpinyl acetate (2.96%), trans-caryophyllene (4.48%), alpha -humulene (3.98%), cedrol (5.65%), myrecene(5.88%), beta pinene (2.67%), sabinene(1.39%), germacrene D (1. 08%) and bornyl acetate (1.00%). In present investigation antidermatophytic activity of *T. orientalis* essential oil was also screened against selected dermatophytes and *candida albicans* through disc diffusion technique and minimum inhibitory concentration evaluating method. Maximum zone of inhibition 20± 0.29mm was reported against *Trichophyton rubrum* MTCC 296 (AI=0.67) followed by 14±1.00 mm against *Trichophyton tonsurans* MTCC 8475 (AI=0.7). MIC was ranging from 0.7 μl/ml to>2.5 μl/ml. *Candida albicans* (MTCC 3018) was found to be most resistant strain. *Thuja* essential oil exhibited excellent antifungal activity against all pathogen except *C. albicans.* These results suggest the potential therapeutically effect of *T. orientalis* essential oil against dermatophytic fungi.

Key words: *T. orientalis*, essential oil, dermatophytes, Delta-3-carene,MIC

Introduction:

Thuja orientalis L. (syn. *Biota orientalis Endl*) commonly known as Morpankhi belonging to the Family Cupressaceae is an ever green monoecious shrubs used in various forms of homeopathy and traditional medicines in various methods. It is cultivated as an ornamental tree in cool and moist places for its extremely beautiful dense foliage and bush like habit of growth. The shoot are flat, leaves are scale like. The phytoconstituents of *T. orientalis* such as flavonoids and terpenoids showed the biological activities (1). Biological activities of *T. orientalis* such as antimicrobial(2), antioxident(3), antibacterial (4), anti-inflammatory (5), insecticidal (6), nematicidal (7), anticancer (8) are well studied. Hair growth promoting activity of hot water extract of *Thuja* was studied by Zhang et al (9). Essential oil derived from the *Thuja* have some toxic properties. Ingestion of *Thuja* leave oil even can cause death (10).

Dermatophytoses possess a serious concern to sociologically backward and economically poor population of India. The dermatophytes represent more than 40 closely related species classified in three genera: *Microsporum*, *Trichophyton* and *Epidermophyton*. High humidity and high temperature condition of Jaipur city specially in summer, favour the incidence of fungi and consequently the diseases (11-13). Skin infection due to dermatophytes has become a significant health problem affecting children, adolescent and adults(14). This may be

the result of frequent usage of antibiotics, environmental condition, immuno-suppressive drugs and various conditions, like organ transplantation, lymphomas, leukemia and human immunodeficiency virus (15).

The present investigation deal with the screening of *T. orientalis* leaves essential oil and their antidermatophytic activity against some selected dermatophytes and *Candida albicans*.

Material and Methods

Collection of plant and extraction of oil: *T. orientalis* leaves were collected from Rajasthan University campus. Identification was done by Botanist Prof. S. Misra, Herbarium (Voucher number RUBL 21183), Department of Botany ,University of Rajasthan, Jaipur, India. Leaves were shade dried and cut into small pieces. The semi-crushed leaves were hydrodistilled in a Clevenger's apparatus for 7-8 hours. Essential oil collected in tubes were dried with anhydrous sodium sulphate. Moisture free oil was than stored in amber coloured bottles and kept in the refrigerator.

Gas chromatography : The quantitative analysis of the essential oils were carried out using a Shimadzu GC- 2010. Nitrogen was used as carrier gas at 10 psi inlet pressure with FID and Omega SPTm column (30.0 m x 0.25 mm ID, film thickness 0.25 μm). Injector and detector temperatures were 270°C and 280°C respectively. Column temperature programmed from 80°C (2 mins hold), 80°C to 180°C at 4°C/min and 180°C to 230°C at 6°C/min with hold time of 6 min and 19 min. respectively. The flow rate of carrier gas was 1.21 ml/min and split ratio was 1:80. The data were processed on GC solutions software for oil composition.

GC-MS Analyses : GC-MS data was obtained on a Shimadzu GCMS-QP-2010 plus system using Omega SPTm column (30.0 m x 0.25 mm ID. film thickness 0.25 um). Helium was used as carrier gas. Injector, Mass detector and Ion source temperatures were 270°C, 280°C and 250°C respectively. Column temperature programmed from 80°C (2 mins hold), 80°C to 180°C at 4°C/min and 180°C to 230°C at 6°C/min with hold time of 6 min and 19 min. respectively. The flow rate of carrier gas was 1.21 ml/min and split ratio was 1:80. EI source and mass range were 70 eV and 40-850 amu respectively. Compounds were identified by using Willey, NIST and Perfumery libraries.

Fungal culture: For antidermatophytic studies five fungi and one yeast procured from the Imtech Chandigarh are *Trichophyton rubrum* (MTCC 296), *T. mentagrophytes* (MTCC 7687), *T. tonsurans* (MTCC8475), *Microsporum canis*(MTCC2820), *Candida albicans* (MTCC3018) and *Microsporum fulvum*(MTCC2837). These selected fungi are maintained on Sabouraud's dextrose agar media and Potato dextrose agar media.

Screening of oil of their antifungal activity:

Disc diffusion method: The filter paper disc method Wannisorn *et al.*(16)was used for evaluation of antifungal activity of essential oil. Standard size whatman no. 1 filter paper discs 6.0 mm in diameter, sterilized by dry heat at 140°C in an oven for one hour were used to determine antifungal activity. 20 ml sterilized Sabouraud's dextrose agar medium was taken in each autoclaved petridish and allowed to solidify. Fungal spore suspension was prepared in sterilized distilled water by transferring a loopful of 15 day-old culture. 1 ml of spore suspension of approximately 0.5 to 5×10^4 (cfu/ml) was spread over the respective agar medium plates. Sterilized filter paper were soaked in neat undiluted oil. An oil saturated disc was placed on an agar plate containing fungal spore suspension. These plates were incubated at 37ºc for 72 hours. Five replicates were kept in each case and the average values were determined and inhibition zone were observed. Ketoconazole was used as a standard drug. The antifungal activity was determined by measuring the inhibition zone around the disc. The activity of oil was measured by the following formula.

$$\text{Activity Index} = \frac{\text{Inhibition Zone (IZ) of the sample}}{\text{Inhibition Zone (IZ) of the Standard}}$$

Semisolid agar antifungal susceptibility method : Minimum inhibitory concentration was determined by Semisolid agar antifungal susceptibility testing method(17). For this experiment Brain Heart Infusion Agar (Hi-media) was used. BHIA was prepared according to manufacturer's instruction.

Inoculum preparation: Sterile swab dipped into sterile tween 80 was used to pick the pure colony of test organism. This was then suspended in 3-4 mL of sterile normal saline and vortexed. The turbidity of the homogenous suspension was adjusted to ~0.5 McFarland standard.

Inoculation of drug containing tubes : The semisolid agar tubes containing known concentrations of test oils as well as oil-free controls, prepared in triplet, were inoculated with one loopful (HimediaFlexi loop 4) of 0.5 McFarland adjusted culture by inserting the loop deep within the semisolid agar. A loopful of the inoculum suspension was streaked onto Sabouraud dextrose agar to check for purity and viability. The tubes were incubated at 37°C for 72 hours

End point determination: End point determination was carried out according to the NCCLS/CLSI guidelines, M27-A and M38-A. Growth was compared to that of oil-free control and scored by visual inspection as follows : +4: growth same as control; +3: slight decrease in growth; +2: significant reduction in growth reduction 80% in yeast and 50% in filamentous); +1 slight growth or few visible hyphal fragments; 0: no growth.

Statistical analysis: Each parameter was tested in triplicate. Conventional statistical methods were used to calculated means and standard deviations. Statistical analysis (T-test) was applied to the data to determine differences ($p < 0.05$).

Results and Discussion

GC/ GC-MS analysis of *T. orientalis* essential oil showed the presence of 38 compounds for 100% of total oil which listed in their elution(Fig.1,2). Delta-3-carene (30.37%) was the main component of *Thuja* oil followed by alpha -pinene (13.11%), limonene (10.07%), alpha-terpinene (8.44%), alpha -terpinyl acetate (2.96%), trans- caryophyllene (4.48%), alpha -humulene (3.98%), cedrol(5.65%), germacrene D (1. 08%) and bornyl acetate (1.00%). Other compounds are alpha thujene (0.75%), alpha-phellandrene (0.59%), alpha -terpine (0.29%), gamma- terpinen (0.55%), terpinen-4-ol (0.76%), alpha -terpineolt (0.33%), ocimenyl acetate (0.61%), beta- elemene (0.20%), beta- cedrene (0.53%), alpha-selinene (0.19%), gamma -muurolene(0.27%), delta-cadinene (0.71%), elemol (0.28%), caryophyllene oxide (0.65%), viridiflorol (0.52%), humulene epoxide II (0.49%), tau- muurolor (0.24%) and alpha-cadinol (0.41%).Similar result was obtained by Nickavar et al.(18). They studied the fruit and leaf essential oils of *T. orientalis*in Iran and found alpha pinene (21.9%)as major constituent of leaf oil followed by alpha cedrol (20.3%), delta 3 carene (10.5%) and limonene(7.2%). Guleria *et al.*(19) studied the essential oil of *T. orientalis* collected from western Himalayan region. Leaf oil contained alpha-pinene (29.2%), delta-3-carene (20.1%), alpha-cedrol (9.8%), caryophyllene (7.5%), alpha-humulene (5.6%), limonene (5.4%), alpha-terpinolene (3.8%) and alpha-terpinyl acetate (3.5%) as major constituents.

Tsiri *et al.*(20) investigated the chemical composition of the essential oils of four varieties of *Thuja*species like *T. occidentalis 'globosa'*, *T. occidentalis 'aurea'*, *T. plicata*and *T. plicata 'gracialis* cultivated in Poland. The main constituents in all samples were the monoterpene ketones *á*- and *â*-thujone, fenchone and sabinene, as well as the diterpenesbeyerene and rimuene. They also checked the antimicrobial activity of these oil against selected bacteria and *Candida* sp. Jirovetz *et al.* (21) studied the chemical composition and antimicrobial activity of *Salva* sp. and *Thuja* sp. essential oils.

Antidermatophytic activity of *Thuja* essential oil was studied by disc diffusion method and minimum inhibitory concentration determination. Maximum Zone of inhibition was found to be 20±0.288mm against *T. rubrum* MTCC 296 (AI=0.67), but maximum activity index 0.73 was

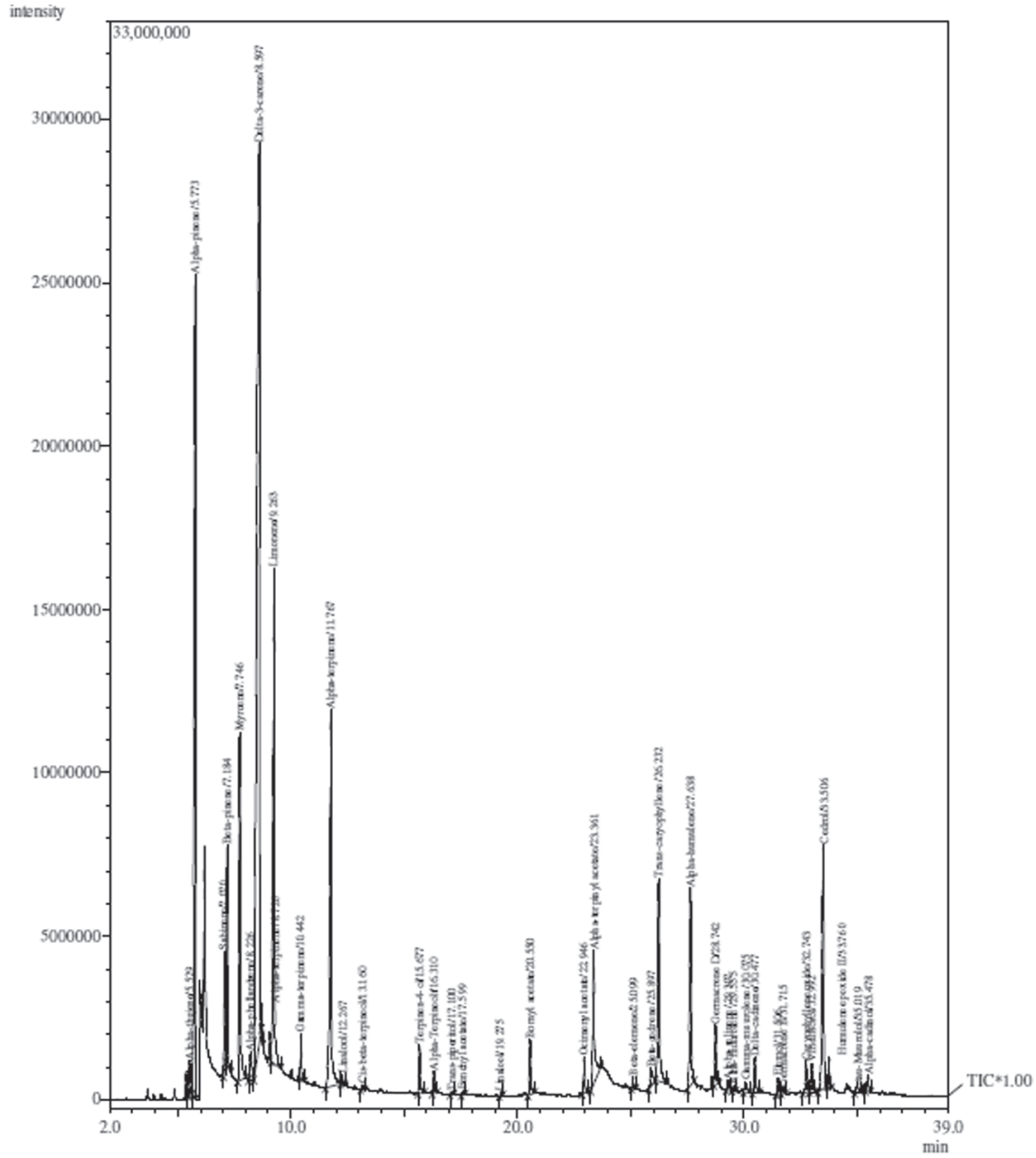

Fig. 1. GCMS analysis of *Thuja orientalis*

Fig. 2. Chemical compositions of *Thuja orientalis*

Peak#	R.Time	Area	Area%	RI	Name
1	5.529	5654678	0.75	926	Alpha-thujene
2	5.773	99279048	13.11	933	Alpha-pinene
3	7.070	10532112	1.39	972	Sabinene
4	7.184	20212491	2.67	975	Beta-pinene
5	7.746	44539565	5.88	992	Myrcene
6	8.226	4463801	0.59	1005	Alpha-phellandrene
7	8.597	229961085	30.37	1014	Delta-3-carene
8	8.726	2169635	0.29	1017	Alpha-terpinene
9	9.263	76250386	10.07	1030	Limonene
10	10.442	4178735	0.55	1058	Gamma-terpinene
11	11.767	63941559	8.44	1089	Alpha-terpinene
12	12.267	983255	0.13	1101	Linalool
13	13.160	563719	0.07	1121	Cis-beta-terpineol
14	15.677	5791080	0.76	1176	Terpinen-4-ol
15	16.310	2495738	0.33	1191	Alpha-Terpineolt
16	17.100	275300	0.04	1208	Trans-piperitol
17	17.599	405468	0.05	1219	Fenchyl acetate
18	19.275	235879	0.03	1257	Linalool
19	20.550	7550788	1.00	1285	Bornyl acetate
20	22.946	4611929	0.61	1341	Ocimenyl acetate
21	23.361	22448055	2.96	1350	Alpha-terpinyl acetate
22	25.099	1494033	0.20	1391	Beta-elemene
23	25.897	3976515	0.53	1410	Beta-cedrene
24	26.232	33937017	4.48	1418	Trans-caryophyllene
25	27.638	30168221	3.98	1453	Alpha-humulene
26	28.742	8204553	1.08	1480	Germacrene D
27	29.307	1473653	0.19	1493	Alpha-selinene
28	29.555	550832	0.07	1500	Alha-muurolene
29	30.075	2081752	0.27	1507	Gamma-muurolene
30	30.477	5339706	0.71	1512	Delta-cadinene
31	31.506	2139791	0.28	1526	Elemol
32	31.715	974739	0.13	1528	Germacrene B
33	32.743	4906070	0.65	1542	Caryophyllene oxide
34	32.992	3943967	0.52	1545	Viridiflorol
35	33.506	42806479	5.65	1552	Cedrol
36	33.760	3698369	0.49	1555	Humulene epoxide II
37	35.019	1838680	0.24	1572	tau-Muurolol
38	35.478	3138623	0.41	1578	Alpha-cadinol
		757217306	100.00		

observed against *Microsporum canis* MTCC 2820(IZ=10.3±0.58mm) followed by AI=0.7against *T. tonsurans* MTCC 8475(IZ=14± 1.00 mm) (Table1). Zone of inhibition 10±0.00 mm was observed against *M. fulvum* MTCC 2837 (AI=0.34), 9±0.58mm against *T. mentagrophytes* MTCC 7687(AI=0.36) and 6±0.00 mm against *Candida albicans* MTCC 301(AI=0.21).*Candida albicans* was found to be most resistant strain from *T. orientalis* essential oil.MIC was determined by semi solid agar antifungal susceptibility testing method with visual end point determination according to NCCLS recommendations. Maximum effect was seen against *T. mentagrophytes* MTCC 7687(0.7 µl/ml) followed by *M. canis* MTCC 2820(0.9 µl/ml),*T. rubrum* MTCC 296 (0.9 µl/ml), *M. fulvum* MTCC 2837(1.1 µl/ml) and *Candida albicans* MTCC 3018(>2.5 µl/ml).(Table. 2)

Jain and Garg (2)studied antimicrobial activity of *T. orientalis* essential oil against six bacteria and five plant pathogenic fungi through disc diffusion technique. The oil was found moderately activity against all six bacteria. Maximum 21mm inhibition zone was observed against *Curvularia lunata.* Most of work has been done on antibacterial activity and on plant pathogenic fungi of *Thuja* plant extracts(3-4,22-25). There are no studies on antidermatophytic properties of *T. orientalis* so far and the present study is the new report.

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Table 1: Antifungal activity of *T. orientalis* essential oil and compared with standard drug

Fungi	*C. lemon* IZ	Ketoconazole IZ	AI
*Candida albicans*MTCC 3018	6±0.00	28±0.58	0.21
*Microsporum canis*MTCC 2820	10.3±0.58	14±0.00	0.73
*Microsporum fulvum*MTCC 2837	10±0.00	29.1±1.15	0.34
*Trichophyton rubrum*MTCC 296	20±0.29	30±0.58	0.67
*Trichophyton mentagrophytes*MTCC 7687	9±0.58	25±0.00	0.36
*Trichophyton tonsurans*MTCC 8475	14±1.00	20±0.58	0.70

IZ = Inhibition zone including 6 mm diameter of filter paper disc;
AI=Activity Index

Table 2 : MIC (µl/ml) of essential oils against selected dermatophytes

Test Organisms	MIC
1. *Candida albicans* MTCC 3018	>2.5
2. *Microsporum fulvum*MTCC 2837	1.1
3. *Microsporum canis*MTCC 2820	0.9
4. *Trichophyton rubrum*MTCC 296	0.9
5.*Trichophyton mentagrophytes*MTCC 7687	0.7

Conflict of Interest: No conflict of interest.

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Stability Indicating Assay Method for the Determination of Medroxy Progestrone Aceate in Bulk Drug and Formulation by HPTLC

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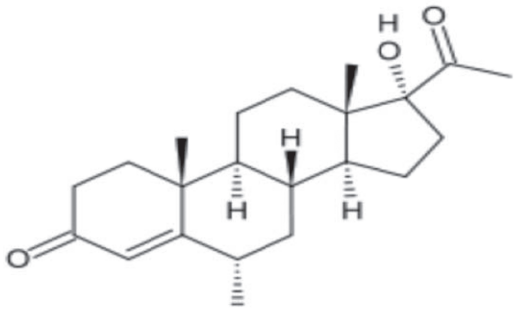
Abstract

A sensitive, accurate, precise and stability indicating high-performance thin layer chromatographic assay method for analysis of Medroxyprogestrone acetate (MPA) in both bulk drug and formulation was developed and validated. The method employed HTLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of Toluene: ethyl acetate: ammonia solution (80:20:0.1 %v/v). This system was found to give compact spots for MPA (*R*f value 0.32±0.02). MPA was subjected to acid, alkali and neutral hydrolysis, oxidation, sunlight and dry heat treatment. The degraded products were well separated from the pure drug with notably different R_f values. CAMAG HPTLC instrument was used for chromatographic separations. Densitometric analysis of MPA was carried out in the absorbance mode at 240 nm. The linear regression data for the calibration plots showed good linear relationship with correlation coefficient 0.9998 ±/0.001 in the concentration range of 50.0-1800.0ng spot^{-1}. The value of slope and intercept were 11.68 and 73.414 respectively. The method was validated for precision, accuracy, robustness, and recovery. The limits of detection and quantitation were 16.5 and 50.0 ng spot^{-1} respectively.

1. Introduction

MPA is a progestin, and is a common component of hormonal contraceptives. It can be used in the treatment of abnormal menstrual bleeding or amenorrhea. Chemically, MPA is 17-Acetyl-17-hydroxy-6,10,13-trimethyl-1,2,6,7,8,9,10,11,12,13,14,15,16,17 – tetradecahydrocyclopenta phenanthren-3-one (Fig.1) with a molecular weight of 344.488 g/mol.

The present study describes development and validation of a simple sensitive, specific, and precise stability indicating HPTLC method for the estimation of MPA in bulk drugs and pharmaceutical formulations. The International Conference on Harmonization [ICH] (1) guidelines emphasizes the stress testing of the drug substance which can help to identify the likely degradation products and validate the stability indicating power of the analytical procedures used.



Medroxyprogesterone

Fig. 1. Structure of Medroxyprogestrone

Chromatographic techniques are widely used for the stability studies as it can be efficient tools to separate and quantify the analyte in presence of decomposition products. The advantage of HPTLC is that several samples can be run simultaneously by using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost. The aim of the present work was to develop an accurate, specific, and reproducible stability indicating HPTLC method for the determination of MPA in the presence of degradation products under stress testing in Bulk drugs and Pharmaceutical formulations as per ICH guidelines (1-2).

The parent drug stability test guidelines (Q1A R2) issued by International Conference on Harmonization (ICH) requires that analytical test procedures for stability samples should be fully validated and the assays should be stability indicating. Stress testing is a part of development strategy under ICH requirements and is carried out under more severe conditions than accelerated studies. Further, it is suggested that stress studies should be carried out to establish the inherent or intrinsic stability characteristics of the molecule by establishing the degradation pathways and help in validation of the analytical methods to be used in stability studies.

The primary goal of the present study was to develop and validate a HPTLC method as per ICH guidelines (3). A second objective was to develop a stability indicating technique, which could be employed for the routine quantification of low levels of medroxyprogestrone in the presence of degradation products for assessment of purity of the bulk drug and stability of its bulk formulations (4).

Structural information on drug degradants and impurities can serve to accelerate the drug discovery and development cycle (5). Forced degradation studies are used to facilitate the development of analytical methodology, to gain a better understanding of active pharmaceutical ingredient and drug product stability (6). The available regulatory guidance provides useful definitions and general comments about degradation studies. Forced degradation studies are carried for development and validation of stability indicating methodology, determination degradation pathways and structural elucidation of degraded products [7]. The objective of stability testing is to determine for what time period and under what condition the product is satisfactory (8). Pharmaceutical companies perform forced degradation studies during preformulation to help select compounds and excipients for further development, to facilitate salt selection or formulation optimization, and to produce samples for developing stability indicting analytical methods (9).

In one of the literature stability indicating analytical method was reported for the determination of medroxyprogestrone in bulk drug and pharmaceutical dosage form by HPLC (10).

Till to day no article related to the stability indicating high-performance thin layer chromatographic (HPTLC) determination of MPA in bulk drug and pharmaceutical dosage forms was reported in literature or in Pharmacopoeias.

Experimental

Materials: MPA bulk drug and its injectable suspension were given by Star drugs and research laboratories, Bangalore as a gift sample. Ethyl acetate, Toluene, methanol and ammonia solution (Qualigens Fine Chemicals, Mumbai) used were of analytical grade, camag linomat IV sample applicator equipped with 100ml Hamilton (USA) syringe, Camag twin trough glass chamber, CAMAG HPTLC scanner III densitometer, Cats 3 software.

HPTLC instrumentation: The samples were applied in the form of bands on the plate, width 6 mm, and 10mm from the bottom of the edge using a Camag precoated silica gel aluminium plate 60F-254 (20 × 10 cm with 0.2 mm thickness, E.Merck, Germany) with Linomat IV (Switzerland) sample applicator equipped with a 100µL Hamilton (USA) syringe. A constant application rate of 100nL/s was employed and the standard, sample volume was 10µL, the space between two bands were 7

mm and slit dimension was kept 5 mm x 0.45 mm micro and 5 mm sec^{-1} scanning speed was employed. The eluting solvent was consisted of Tolune: ethyl acetate: ammonia solution (80:20:0.1 % v/v). Linear ascending development was carried out in twin trough glass chamber (Camag) saturated with mobile phase. Previously the glass chamber was saturated with help of filter paper and the optimized chamber saturation time was found to be 30 min at room temperature. The length of chromatogram run was approximately 80 mm. After the development the plates were dried in air with the help of an air-dryer. Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 240 nm with CATS3 software. The source of radiation utilized was a deuterium lamp. The slit dimensions were 5mm x 0.45 mm micro and the scanning speed was 5 mm sec^{-1}.

Stock Preparation

Preparation of stock solution: Stock solution of 1mg/ml was prepared by dissolving 10 mg of MPA in 10 ml of methanol. From this 5.0, 10.0, 20.0, 40.0, 50.0, 100.0, 135.0 and 180.0µg mL^{-1} solutions were prepared in methanol. 10.0µl of each of this solution were spotted on the HPTLC plate to obtain concentrations of 50.0, 100.0, 200.0, 400.0, 500.0, 1000.0, 1350.0 and 1800.0ng $spot^{-1}$ of MPA respectively.

Method validation

***Calibration curve of* MPA:** The working standard solution (mentioned in section 2.3.1) each of 10 µl were applied on HPTLC plate to obtain concentration of 50.0, 100.0, 200.0, 400.0, 500.0, 1000.0, 1350.0 and 1800.0ng $spot^{-1}$ of MPA. The curve was plotted using peak area against the drug concentrations and the data was treated by the linear least square regression. The range was chosen based on the maximum concentration to be kept for the stress studies.

Accuracy and precision of the assay

Precision: Repeatability of sample application and measurement of peak area were carried out using six replicates of the same spot (500 ng $spot^{-1}$ of MPA). The intra-day and inter-day variation for the determination of MPA was carried out at a concentration of 500 ng $spot^{-1}$.

Accuracy: The analysed samples were spiked with extra 80, 100, 120 % of the standard MPA and the mixture were analysed by the proposed method. At each level of the above said amount, three determinations were performed. This was done to check the recovery of the drug at different levels in the formulations. Recovery was calculated using the standard formula

Recovery [%] = [(Total conc.

ÿ Formulation conc.)/ Standard conc.] × 100.

Repeatability

Repeatability of measurement of peak area: MPA (500 ng/spot) of 10 µl was spotted on a HPTLC plate, developed, dried and the spot was scanned seven times without changing the plate position and % RSD for measurement of peak areas were estimated.

Repeatability of measurement of sample applications : The drug solution, MPA, 50 µl (500 ng/spot) was spotted seven times on a HPTLC plate, developed and dried. The spots were scanned and RSD% for measurement of peak areas was estimated.

Robustness: By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition of Toluene: ethyl acetate: ammonia solution (78:22:0.1 and 78:22:0.1 v/v) were tried and samples were run. The mobile phase composition, chamber saturation time and temperature for activation of plate were tried in the varied range (25 and 30 min at 100°C and 120°C respectively). Robustness of the method was done at concentration level of 500 ng $spot^{-1}$.

Limit of detection and limit of quantification: In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times following the same method as explained in Section 2.2. The signal-to-noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1.

Specificity: The specificity of the method was ascertained by analyzing standard drug with sample. The spot for MPA in sample was confirmed by comparing the R_f values and spectra of the spot with that of standard. The peak purity of MPA was assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M) and peak end (E) positions of the spot.

Analysis of MPA in prepared formulation: To determine the concentration of MPA in sterile injectable suspension (labeled claim: 150 mg mL^{-1}), 100 µL (15 mg) of MPA injectable suspension was pipetted out and transferred into three 10 mL volumetric flasks. 5.0 mL of methanol was added into each of the flasks, sonicated to dissolve the contents and then the volume was finally made up with methanol. Further this solution was diluted appropriately using methanol to obtain a concentration of 100.05 µg/mL (67 µL to 1000 µL), from this solution further diluted 100 µL to 1000 µL with methanol, from this solution 50µL (500 ng $spot^{-1}$) was applied on HPTLC plate followed by development and scanning as described in section 2.2. The analysis was repeated in triplicate. The possibility of excipients interferences in the analysis was studied.

Forced degradation of MPA: All stress degradation studies were performed at initial concentration of 100µg mL^{-1}. For these studies, 10 mg of MPA was accurately weighed and transferred to a 100 mL volumetric flask (step one).

Acid and base induced degradation: To the step one, 1mL of each 0.01N NaOH (for Alkaline degradation), 0.1N HCl (for Acid degradation),) were added to separate volumetric flasks and dissolved the content and diluted with methanol to make up the volume and prepared solution were placed at room temperature for 2 hrs for alkaline degradation and then refluxed at 80°C for 8 hrs.

The mixtures were allowed to cool and 1.0 mL of these solutions were then transferred to a 10 mL volumetric flasks neutralized with 1.0 mL of 1N NaOH for acid and neutralized with 1.0 mL of 1N HCl for alkaline degradation and the volumes were made up with methanol. From this solution 50µL and 2 x 50 µL samples were plotted in the form of bands by using Linomat IV, Switzerland applicator on precoated silica gel aluminium plate 60GF-254 to get 500ng/spot and 100ng $spot^{-1}$ respectively for MPA.

Hydrogen peroxide-induced degradation, Neutral Hydrolysis, Sunlight and Dry heat degradation.

To the step one, 1.0mL of 3 % Hydrogen peroxide (for Oxidative degradation) and 1.0 mL of water (for Hydrolytic degradation) were added in two separate volumetric flasks and dissolved the content and diluted with methanol to make up the volume and placed at room temperature for 8 hrs and refluxed at 80°C for 8 hrs respectively . Additionally, the drug powder in step one was exposed to dry heat at 80 ºC for 8 hrs and at sunlight for 8 hrs.

The mixtures were allowed to cool and made up to the mark with the diluent. 1.0 mL of this solution was then transferred to a 10 mL volumetric flask and the volume was made up with methanol. From this solution 50µL and 2 x 50µL samples were plotted in the form of bands by using linomat IV applicator on precoated silica gel aluminium plate 60GF-254 to get 500ng $spot^{-1}$ and 100ng $spot^{-1}$ respectively for MPA.

Results and Discussion

Method development

Development of mobile phase: HPTLC procedure was optimized with a view to developing a stability indicating assay method. Three solvent ratios were selected as to optimize the best among them. Initially, Tolune: ethyl acetate (80:20 v/v) gave good resolution with R_f value of 0.32 for MPA but typical peak nature was missing. Finally, the mobile phase consisting of toluene: ethyl acetate: ammonia solution (80:20:0.1 v/v %) gave a sharp and well defined peak at R_f value of 0.32 (Fig. 2). Well-defined spots were obtained when the chamber was saturated with the mobile phase for 30 min at room temperature.

Validation of Method

Calibration curves: The linear regression data for the calibration curves showed a good linear relationship over the concentration range 50-1800 ng spot^{-1} with respect to peak area. The regression-coefficient (r^2) was found to be 0.9998 which is within the acceptance criteria limit of ≥ 0.99.

Precision: The repeatability of sample application and measurement of peak area were expressed in the terms of % RSD and the results are depicted in Table 1, which revealed intra-day and inter-day variation of MPA at concentration level of 500 ng spot^{-1}.

Robustness of the method: The standard deviation of peak areas was calculated for each parameter and %R.S.D. was found to be less than 2%. The low values of %RSD values as shown in Table 2 indicated robustness of the method.

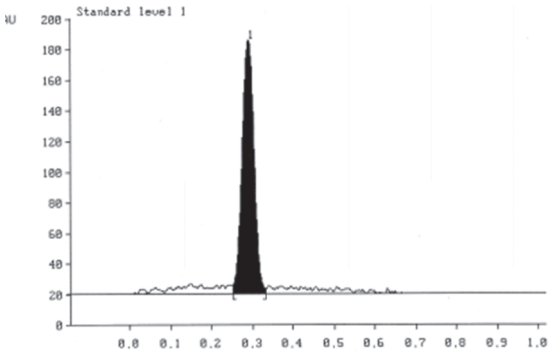


Fig. 2. A typical HPTLC chromatogram of MPA (500 ng spot -1) (Rf = 0.32).

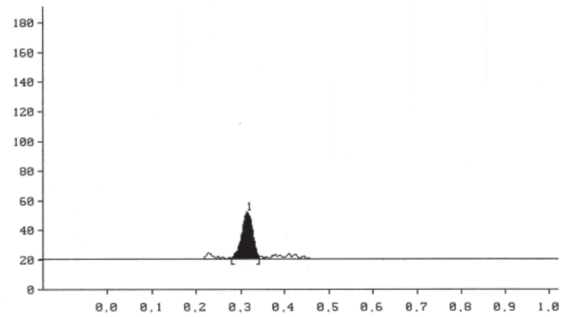


Fig. 3. HPTLC chromatogram of MPA at the LOQ (50 ng spot -1).

LOD and LOQ: The signal-to-noise ratio 3:1 and 10:1 were considered as LOD and LOQ, respectively. The LOD and LOQ were found to be 16.5 and 50 ng spot^{-1}, respectively. Chromatogram at LOQ of MPA is given in fig. 3.

Specificity: The developed method was used for the estimation of MPA in bulk drug and pharmaceutical dosage form. The excipients did not interfere in the estimation. Interferences from the formulation and degradants were absent. These results indicated the specificity of the method.

Recovery studies (Accuracy): The proposed method when used for extraction and subsequent estimation of MPA from pharmaceutical dosage forms after spiking with 80, 100 and 120% of additional drug afforded mean recovery of 100.09 ± 0.93 as listed in table- 3. The data of summary of validation parameters are listed in table-4.

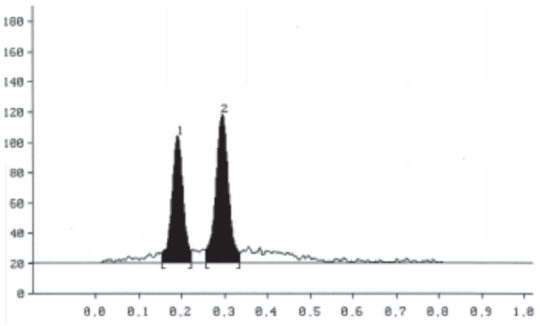


Fig. 4. HPTLC chromatogram of alkali degraded MPA.

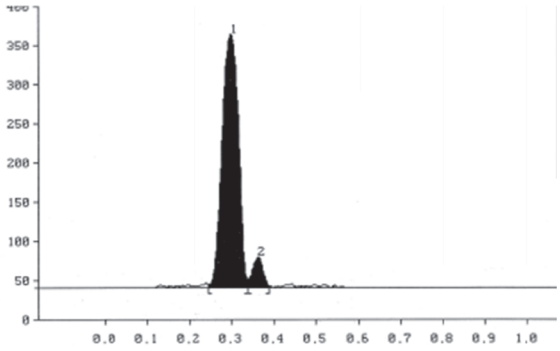


Fig. 5. HPTLC chromatogram of hydrogen peroxide degraded MPA.

Table 1. Intra- and inter-day precision of HPTLC method

Amount (ng/spot)	Intra-day precision [a]			Inter-day precision [a]		
	Mean area	S.D.	RSD%	Mean area	S.D.	RSD%
500	6106.9	52.36	0.86	6086.7	23.01	0.38

[a] n = 6.

Table 2. Robustness of the method

Parameter	**S.D. of peak area**	**RSD%**
Mobile phase compositionToluene: Ethylacetate:Ammonia (78:22:0.1)	2.303	0.463
Mobile phase compositionToluene: Ethylacetate:Ammonia (82:18:0.1)	0.981	0.196
Chamber saturation time (25 min)	0.883	0.177
Chamber saturation time (35 min)	0.602	0.120
Temperature for activation of plates (100°C)	1.656	0.333
Temperature for activation of plates(120°C)	0.646	0.129

Table 3. Recovery studies[a]

Recovery level (%)	**Amt of drug added (µg/ mL)**	**Amount of drug found (µg/ mL)**	**Percentage recovery**	**Mean % recovery**	**RSD%.**
80	400.000	407.794	101.95	101.12	0.73
	400.000	403.571	100.89		
	400.000	402.059	100.51		
100	500.000	497.384	99.48	99.29	0.16
	500.000	495.903	99.18		
	500.000	496.051	99.21		
120	600.000	600.571	100.10	99.87	0.26
	600.000	597.520	99.59		
	600.000	407.794	101.95		

Mean Recovery (Mean±S.D.)= 100.09 ± 0.93
[a] (n=9)

Analysis of prepared formulation: A single spot of R_f 0.42 was observed in chromatogram of the MPA samples extracted from sterile injectable suspension. There was no interference from the excipients commonly present in the formulation. The average MPA content was found to be 99.33% with ± SD of 0.28 and listed in Table 5. Therefore it may be inferred that degradation of MPA had not occurred in the formulation which were analysed by this method. The low %RSD value indicated the suitability of this method for routine analysis of MPA in pharmaceutical dosage forms.

Table 4. Summary of validation parameters

Parameter	Data
Linearity range (ng spot^{-1})	50-1800
Correlation coefficient	0.9998
Limit of detection (ng spot^{-1})	16.5
Limit of quantitation (ng spot^{-1})	50
Percent recovery (n = 9)	100.09 ± 0.93
Precision (RSD%.)	
Repeatability of application (n = 6)	0.96
Repeatability of measurement (n = 6)	0.07
Inter-day (n = 6)	0.86
Intra-day (n = 6)	0.38
Robustness	Robust
Specificity	Specific

Stability- indicating property: The chromatogram of samples degraded with acid, base, hydrogen peroxide, neutral, sunlight and heat showed well separated spots of pure MPA as well as some additional peaks at different R_f values. The spots of degraded products were well resolved from the drug spot as shown in Figs. 2-6. Percentage degradation was calculated and listed in table-6.

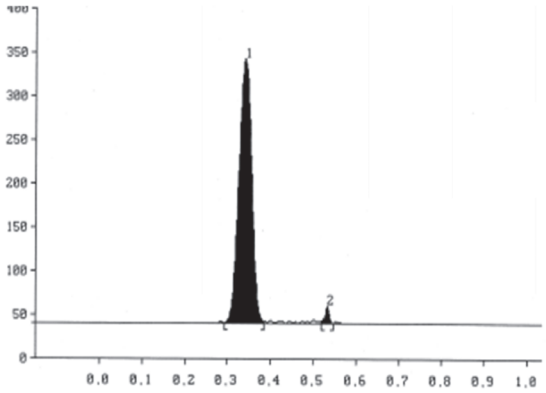


Fig. 6. HPTLC chromatogram of sun light degraded MPA

Table 5. Analysis of MPA sterile injectable suspension (500.00 ng spot^{-1})

Pharmaceutical formulation	Actual concentration of MPA (ng spot^{-1})	Amount of MPA calculated (ng spot^{-1})	% MPA (mean±SD) (n=3)
MPA Sterile injectable suspension	500.000	496.678	99.33±0.28

Table 6. Forced degradation of MPA

S.No.	Stress Condition	% Degradation ± S.D.
1	Base(0.01 N NaOH) @ RT for 2 Hour	24.91 ± 0.41
2	Acid(0.1 N HCl)@ 80°C for 8 Hour reflux	6.33 ± 0.11
3	Oxidative (3% H_2O_2) RT for 8 Hour	4.96 ± 0.26
4	Thermal (80°C) for 8 Hour	4.11 ± 0.02
5	Neutral (water)@ 80°C for 8 Hour	4.70 ± 0.03
6	Sunlight for 8 Hour	3.03 ± 0.13

Conclusion

The new HPTLC method is specific, and sensitive for the estimation of MPA. Statistical analysis proves the method is repeatable and selective for analysis of MPA. The proposed method is less time consuming and it can be used for routine Quality control test and stability studies of MPA in Bulk drug and in its pharmaceutical formulations. As the method separates the drug from its degradation products, it can be employed as a stability indicating one.

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Cloud Computing and its Need in Life Science's R & D

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Abstract

The cloud computing is driven by factors such as aging of the current information technology (IT) infrastructure and changes in IT landscape. Virtualization and cloud computing have transformed the way the IT services are delivered to customers at a lower cost. The research and development (R&D) in life sciences is on cusp of change. The new opportunities and challenges must focus on renewing the existing systems and processes for efficacy and adopting advancements in new technologies to gain value. This paper briefly outlines various cloud computing trends in life sciences.

Introduction

Cloud computing means services carried over internet from anywhere, any place, anybody and any device. A new era is set for an integrated development and the convergence of various technologies such as social media, advanced analytics, digital media, and Big Data with the cloud and explosion of digital data availability due to this convergence play a key role in R&D in life sciences (1, 2). The cloud computing solutions, especially software-as-a-service (SaaS), are reshaping the service models in life sciences. The disruptive technology is considered as the game-changer for life sciences (1, 2). Ultimately, several companies using 'SaaS-first' approach for R&D initiatives and eventually, an "all-in" cloud SaaS scenario will open up promising opportunities related to innovations in drug developments and personalized medicines (1, 2).

Cloud provides three types of services to its users *viz.*

a) Software as a Service (SaaS) – enables users to run applications online through cloud computing techniques Ex: Google mail, Google calendar API, salelsforce.com etc.

b) Platform as a Service (PaaS) – enables the users to build their own cloud applications using supplier-specific tools and languages through cloud computing techniques. Ex:MSSQL, Appche, Linux etc.

c) Infrastructure as a Service (IaaS) – enables the users to run any application on supplier's cloud hardware. Ex: Amazon EC2, GoGrid etc.

Shared resources environment in cloud offers faster computation, significant economies of scale to cut cost and improve operational agility. Cloud computing provides opportunities to move away from capital investments to operational costs (3). Cloud solutions are generally architected for multi-tenancy, scalability and reliability. Instead of buying the big servers, computers, and software packages, cloud computing, like electricity, becomes an operational cost and any analysis can be performed in a few weeks for a few thousand dollars and also moving away from desktop installed packages for services (3). As cloud offers inter organizational or departmental collaboration, migrating to cloud can be a strategic differentiator especially for pharmaceutical companies (3).

The innovation through cloud in life sciences is due to

a. Data accessibility
b. Low cost
c. Infrastructure provisioning
d. More computing power
e. Enhanced collaboration

R&D informatics, social media analytics and digital health technology are creating paradigm shift in life sciences. Cloud computing offers the use of data encryption, password protection, secure data transfer, processes' audits, and the implementation of respective policies against data breeches and malicious use. There is concern and flawed assumption about data privacy and security in public clouds but there is a need to focus more on developing enterprise cloud strategy which includes security in terms of security -aware application design, application self-protection besides traditional measures than worrying about it. Many standards have evolved over a period of time to mitigate the risks. The standard bodies like National Institute of Standards and Technology (NIST), Open Cloud Computing Interface (OCCI), Open Cloud Consortium etc., are working on issues such as data security and data integration (3).

Gartner Inc.,(USA) predicted four outcomes for postmodern business applications, using the hybrid reality, on-premises monolith, outsourced everything, and flip (HOOF) model (Fig. 1). Hybrid is complementing the existing on-premises business applications with cloud-delivered business applications and Flip is "Flipping" the majority of on-premises business applications to the cloud.

Cloud computing trends

The cloud computing in Life Sciences R&D is primarily driven by a surge in multistate clinical trials, wide prevalence of chronic diseases in major regions, and researches in gene therapy and the innovations in drug making such as those of personalized medicines (2, 3). The data explosion due to the rise of genomics has accelerated the adoption of cloud computing in R&D. In addition to the genomic data explosion, the harnessing of real world data from wearables are contributing to the massive data processing

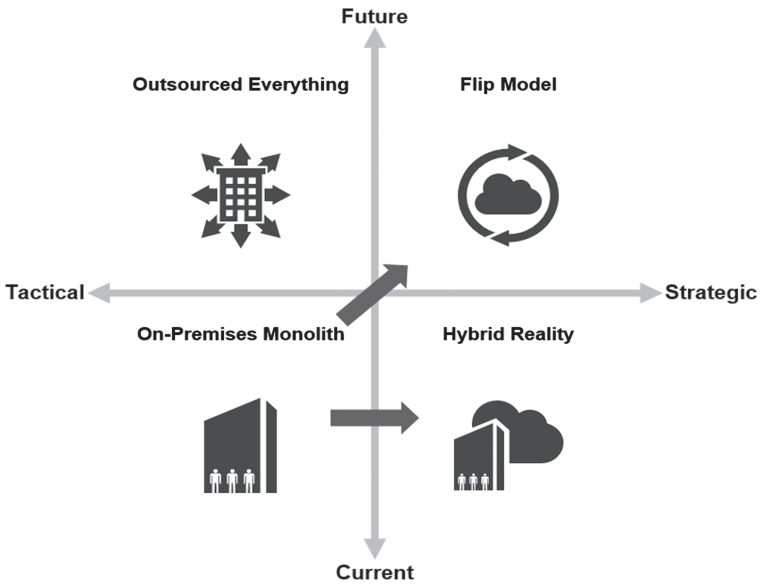

Fig. 1. Gartner's HOOF Model-Options for cloud deployment of business applications
Source: Gartner Inc (August 2016) (with permission from Gartner Inc)

needs which make cloud computing critical. Therefore, adoption of cloud in life sciences enables new ways of working by capturing new opportunities and by transforming life science industry for improving the quality of life (2, 3).

Cloud computing in bioinformatics

It was thought in the beginning that by generating huge amounts of data, it is possible to process and compute the DNA variants by comparing them to the reference genome but storing terra bytes of data and using it for analysis at a later date for updating the variant profile is a huge challenge. Data interpretation and integration is the key in innovation (4-7). Modern biology faces challenges in the field of data storing, management and analysis. Sequencing and cataloguing genetic information has increased many folds (4-7). Various medical research institutes are continuously targeting on sequencing of millions of genomes for the understanding of biological pathways and genomic variations to predict the cause of the disease and developing genome-based therapeutics. The explosion of biological data (the scale of the data exceeds a single machine) has made it more expensive to store, process and analyze compared to its generation. This has stimulated the use of cloud to avoid large capital infrastructure and maintenance costs. The chief advantage to a researcher in biology using cloud computing is the capacity to scale the analysis up and complete it in short period of time. Due to explosion of data, the logic of analysis will be taken near to data and not the other way round. DNA sequencing generates massive amount of data, and its interdisciplinary nature employs cloud computing and big data technologies in life sciences. Next generation cloud based computational biology has the potential to revolutionize life sciences (4, 5,6, 7). Genomics research can lead to a greater height by leveraging the advantages that the current technologies offer. The solutions in cloud computing are expanding rapidly day by day in computational biology (4, 5,6, 7). Cloud computing is currently utilized for:

a. bioinformatics workflows
b. comparative genomic studies
c. gene set analysis for biomarkers
d. identification of epistatic interactions between single-nucleotide polymorphisms
e. microbial/animal/plant sequence analysis
f. multiple sequence alignment algorithms
g. protein annotations and analysis
h. and systems biology

Despite the wide range of different cloud computing platforms available, most of the existing works in computational biology are focused on Amazon Web Services (AWS) as provided by Amazon, Amazon Web Services (AWS). Amazon provides a centralized cloud of public data sets (e.g. archives of GenBank, Ensembl databases, etc.) of biology; chemistry etc., as services and it is the biggest cloud provider for big data processing. Beijing Genomics Institute (BGI), the world's largest genomics organization, introduced its latest-generation cloud-based Software as a Service (SaaS) solution, EasyGenomics™. EasyGenomics integrates various popular next generation sequencing (NGS) analysis workflows including whole genome re-sequencing, exome re-sequencing, RNA-Seq, small RNA, *de novo* assembly, among others (4, 5). EasyGenomics can be accessed via www.easy genomics.com. Galaxy is an open source next generation sequencing platform. Similarly, NextBio is a search tool to mine all the public databases available. DNAnexus is another cloud-based system for NGS data storage and analysis (4, 5). Sage Bionetworks has developed an open source synapse platform for managing and sharing data and computational workflows. Synapse is a platform for supporting scientific collaborations centered on shared biomedical data sets. The primary goal is to make biomedical research more transparent, reproducible, and accessible to a broader audience of scientists.

Cloud computing in pharmaceutical industry

The Research and Development process in pharmaceutical industry is complex and there are many specialized applications of different brands supporting R&D. These applications, which are in silos, generate huge amount of data which

require maintenance due to Federal Drug Agency (FDA) regulations. The companies are looking for ways to reduce the IT budget and hence, are front runners of cloud adoption (2, 3). There is a growing trend to simplify platforms and solutions in areas such as Enterprise resource planning (ERP) environment and non-ERP systems. Typical pharmaceutical applications in bioinformatics such as DNA and protein sequence analysis, machine learning, data mining and applications in chemical informatics such as virtual screening, Quantitative Structure Activity Relations (QSAR) etc., are available as SaaS in cloud. Molecular modeling and simulation, Bio Assay, Basic Local Alignment Search Tool (BLAST) etc., along with other enterprise applications such asERP, Customer Relationship Management (CRM) are hosted in cloud. Besides, a switch to cloud-based solutions in clinical IT has been taking place over the last two-to-four years. Players like Johnson and Johnson (J&J), Pfizer companies have already adopted cloud and started porting their data to cloud.

Cloud computing in agriculture (Mobile agriculture)

Low yield per hectare, accelerated agricultural growth, sustainable water management, combating dry land salinity, poor access to proper infrastructure, credit and modern technology etc., have created new challenges in agricultural farming. In the beginning, Information and Communication Technology (ICT) was adopted to reduce the digital divide in rural populations. But, providing service on demand has pushed the industry and scientists to share knowledge and collaborate which has resulted in adoption of cloud. Spread of wireless networks, low cost mobile phones and high reach of Wi-Fi networks at rural areas made it easy to adopt to cloud. Mobile agriculture (M-agriculture) is playing a crucial role in information gathering and analysis. United States Department of Agriculture (USDA) has transitioned 1,20,000 federal workers from on-premise messaging and collaboration to Microsoft's cloud computing solution with a vision to consolidate disparate messaging environments

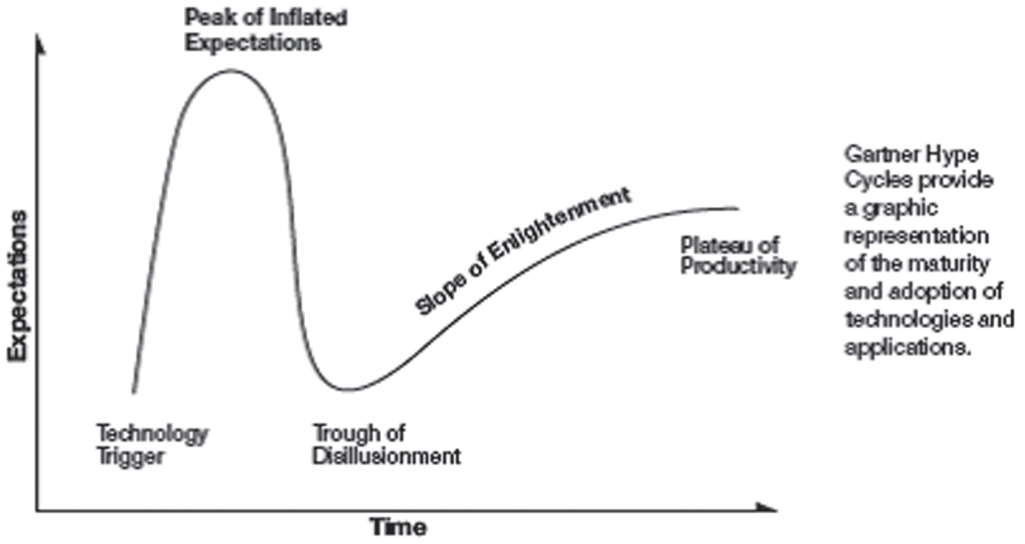

Fig. 2. Hype Cycle– Source: Gartner Inc (August 2016) (with permission from Gartner Inc).

onto a single, unified platform, which will reduce the costs, boost workforce productivity and improve communications and collaboration. International System for Agricultural Science and Technology (AGRIS) is a global public domain database with more than 4 million structured bibliographical records on agricultural science and technology (7). This database is maintained by FAO, and its content is provided by more than 150 participating institutions from 65 countries. Hopefully, it would be more useful to the farming community in the years to come.

The five phases of the life cycle are explained below:

1. Technology trigger - The hype is created by new technology innovation or breakthrough.
2. Peak of inflated expectations - The product generated during this phase may be specialized, costly and have high expectations. More players jump in this phase to understand the fitment and behavior of the new technology in their respective business.
3. Trough of disillusionment - This phase consists of negative publicity and pushing technology beyond its limits.
4. Slope of enlightenment -The technology begins its climb toward the early stages of maturity as second- and third-generation products are launched, and methodologies and tools are added to ease the development process.
5. Plateau of productivity - The plateau of productivity represents the beginning of mainstream adoption, when the business benefits of the technology are positively measured.

The competition that builds up at peak of inflated expectations and the trough of disillusionment may slowly lead to consolidation. Software as a Service (SaaS) is getting more matured and entering into plateau of productivity due to availability of cloud migration tools, cloud testing tools, application PaaSetc (8). The cloud computing and SaaS are the key differentiators for optimizing cost as well as promoting innovation in research (8).

Outlook

Figure 2 describes the hype cycle of cloud computing for the next decade. The emergence of SaaS as a front line model is evident from this figure. AI and machine-learning capabilities are seeping into virtually every technology, and represent a major battleground for technology providers over the next ten years. The next generation may be driven by blending digital and physical worlds with artificial intelligence (AI) and machine learning. It can be assumed that every application or service will incorporate AI in some way over the next 10 years as it can be seen now that some of service providers are increasingly using AI and machine learning in their applications. In life sciences, AI and machine learning are widely used now in advanced analytics. DevOps tools help to overcome the barriers between security and application teams. All future application/service design will embrace DevSecOpsmodel. The future SLA must focus on (a) dynamic storage provisioning in terms of capacity, bandwidth and throughput and coexistence of private and public clouds (b) CPU/ memory virtualization and (c) workflow/service SLAs. CSCC (Cloud Standards Customer Council) is actively working on building the artifacts in leveraging of cloud by various workload types including Big Data, social and mobile. To scale trillions of services in cloud, robust standards in terms of global interoperability and massive scaling are required which may be evolved and defined by standard bodies like NIST, DMTF, SNIA, OGF, IEEE, etc.

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Optimization for Enhanced Production of Antibacterial Metabolites by Marine Actinomycetes *Kocuria* sp. strain rsk4

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Abstract

A new halophilic actinomycetes strain was characterized and on the basis of physiological and 16S rDNA sequence, it was designated as *Kocuria sp*. strain rsk4. It was able to produce antibacterial metabolites and shows broad spectrum antibacterial activity against several gram-positive and gram-negative bacteria. Rsk4 showed highest antagonistic effect against multi drug resistant bacteria *Staphylococcus aureus*. Cultural and nutritional conditions of strain have been optimized under shake-flask conditions for the efficient production of antibacterial metabolites. Among the various factors, starch casein medium, 30ºC temperature, neutral pH, 5% salinity, 7 days incubation period and 180 rpm agitation was found to be optimal for antibacterial metabolite production. 1% starch, yeast extract, casein and magnesium were found to be best carbon source, nitrogen sources and mineral respectively for improved antibacterial activity. Among various combinations of nitrogen source, yeast extract and casein in equal ratio showed enhanced antibacterial activity. This is the first report on the optimization of antibacterial metabolites by *Kocuria* sp. The study suggests that the *Kocuria* sp. could scale up by traditional method for production of antibacterial metabolites effective against multiple drug resistant *S. aureus.*

Key words: Actinomycetes, Antibacterial metabolites, Culture condition, *Kocuria*, Optimization

Introduction

Actinomycetes are potential source for production of valuable bioactive compounds and continue to be routinely screened for novel bioactive substances (1). They are well known for prolific production of commercially important secondary metabolites such as antibiotics (2), antifungal (3), antitumor (4) and immunosuppressive agents (5). Actinomycetes are responsible for production of various antimicrobial substances such as aminoglycosides, anthracyclines, glycopeptides, beta-lactams, macrolides, nucleosides, peptides, polyenes, polyesters, polyketides, actionomycins and tetracyclines (6). Emerging problem of multi drug resistance and new phyto-pathogens in the few decades leads to increasing interest for isolation of actinomycetes from relatively unexplored region (7). Extremophilic actinomycetes are always proved to be promising source for antimicrobial compounds due to adaptation under extreme conditions (8). They have diverse community structure and various unexplored metabolic pathways existing in various genera. Exhaustible study of terrestrial actinomycetes leads to re-isolation of known antimicrobial compounds; hence alternatively marine ecosystems are viewed as a rich source of microorganisms capable of producing unknown novel antimicrobial compounds. Actinomycetes derived from marine habitats provide industrially important secondary metabolites and still considered as a potential source of unique and novel chemical structures (9). Most of the

antibiotics are extracellular metabolites which are normally secreted in culture media (10) and have been used as various drugs. It has been observed that production of antibiotics is not a fixed property of organisms as it is variably affected by various factors such as physical, chemical or genetic composition of organism. Productions of secondary metabolites by microorganisms are dependent on the species and strains of microorganisms and their nutritional and cultural conditions (11). Minor manipulation in media composition exerts a huge impact on quantity and quality of secondary metabolites and general metabolic profile of microorganisms (12). Therefore, designing an appropriate fermentation medium is of critical importance in the production of secondary metabolites (13). Complete knowledge of optimal conditions with particular reference to the strain must be required for maximum fermentation activity leading to production of antimicrobial compounds by actinomycetes (14).

Several statistical and non statistical methods are available for optimization of medium constituents. The problem of statistical approach is selections of media components which are either decided by borrowing or by random selection and too many options are available if the organism under analysis has not been previously studied for production of desired product (15). Optimization of culture medium and physical parameter is efficiently done by non statistical one factor at a time method (16). The aim of the present study was to optimize the conditions for improved antibacterial compound production in the means of antibacterial activity.

Materials and Methods

***Collection and identification of organism*:** The strain rsk4 was isolated from marine water sample collected from Porbandar region (21°37'48"N 69°36'0"E) at Gujarat, Western India. The identification of the strain was carried out on the basis of cultural, morphological and biochemical characteristics. 16S rRNA gene sequencing was also carried out for the molecular identification of microorganism.

Antibacterial activity of strain rsk4: Strain rsk4 was grown in starch casein media with 5% NaCl and antibacterial activity of the ethyl acetate extract was assayed for 10 days by agar well diffusion method on Mueller Hinton agar. Ten pathogenic gram positive and gram negative bacteria were tested: *Staphylococcus aureus* MTCC 96, *Mycobacterium smegmatis* MTCC 6, *Bacillus subtilis* MTCC 441, *Bacillus megaterium* MTCC 2444, *Enterococcus faecalis* MTCC 439, *Klebsiella pneumoniae* MTCC 109, *Proteus vulgaris* MTCC 1771, *Salmonella typhimurium* MTCC 1251, *Pseudomonas aeruginosa* MTCC 2453 and *Escherichia coli* MTCC 739. Zones of inhibition were measured after 24 h incubation at 37ÚC using Hi-Antibiotic ZoneScale (HiMedia). Antibacterial assays were repeated in triplicates to confirm the consistent production of antibacterial metabolites along with media controls. *Staphylococcus aureus* MTCC 96 was found to be more sensitive to the antibacterial agent; hence further studies was carried out with it.

Optimization for production of Antibacterial compound

Effect of Media: Six different media namely starch casein medium (SC), glucose yeast extract malt extract broth (ISP2), inorganic salts starch broth (ISP4), glycerol asparagines broth (ISP5) (17), glucose yeast extract broth (GYEA) (18) and Bennett medium (BM) (19) were inoculated with culture in conical flasks and incubated at 30°C in rotary shaker for 10 days. After incubation, 30 µl ethyl acetate extract was evaluated for antibacterial activity by agar well diffusion method against *Staphylococcus aureus*.

Effect of Incubation Periods: The optimized media was inoculated with actinomycetes strain and incubated for 10 days. 1ml aliquots were withdrawn after every 24 hours and subjected to centrifugation and filtration followed by ethyl acetate extraction. The growth was measured as dry weight per ml of the sample withdrawn to know the relation between growth and product. 30 µl extracts were tested for antibacterial activities by agar well diffusion method against test pathogens.

Effect of Temperature: Rsk4 strain of the actinomycetes was inoculated into optimized media and incubated at different temperature viz. 20, 25, 30, 35, 40, 45 and 50°C for 10 days in rotary shaker. After incubation, ethyl acetate extract were analyzed for antibacterial activities by agar well diffusion method against *S. aureus*.

Effect of agitation: Effect of agitation speed for antibacterial product formation has been studied by inoculation of strain in the optimized media and kept at different agitation speed to achieve high rate of antibiotic production. The different agitation speeds were 60, 100, 140, 180, 220, 240 and 280 rpm. A control flask was maintained at static condition and all the flasks were incubated for 10 days. Antibacterial activity was evaluated by above mentioned method.

Effect of Salinity: Salinity was considered as one of the important parameter for metabolite production. Strain rsk4 was halophilic and isolated initially at 5% NaCl. Optimized media was prepared with different salinity concentrations (0, 2.5, 5, 7.5, 10, 12.5 and 15%) by adding NaCl. The strain was incubated at 30°C for 10 days, growth was measured and antibacterial activity of the ethyl acetate extract was tested against test pathogen *S. aureus*.

Effect of pH: Antibacterial activity of strain at different pH value was measured by agar well diffusion method. The pH of optimized media was adjusted from 5 to10 and incubated at 30°C in rotary condition. 30 µl ethyl acetate extract were used evaluated for its antagonistic effect against *Staphylococcus aureus*.

Effect of Carbon Sources: The effect of different carbon sources on antibacterial metabolite production was studied by replacing carbon source in the optimized medium with other carbon sources namely, dextrose, glucose, sucrose, maltose, mannitol, lactose, glycerol and starch at the concentration of 10 g/L. A flask without any carbon source was kept as a control. The optimized carbon source was varied at different concentration viz. 0.5%, 1%, 1.5%, 2% and 2.5% (W/V) in order to get the high rate of antibiotic production. Antibacterial activity of 30 µl ethyl acetate extract was evaluated by agar well diffusion method against *S. aureus*.

Effect of Nitrogen Sources: Effect of various nitrogen sources was assessed by replacing nitrogen source in the optimized medium with peptone, Beef extract, Yeast extract, Malt extract, casein, ammonium sulphate and potassium nitrate at the concentration of 10 g/L for the maximum antibacterial compound production. A flask without any nitrogen source was kept as a control. Different combination of optimized nitrogen source was combined to get highest antibacterial activity. Antibacterial activity was evaluated by above mentioned protocol.

Effect of Minerals: Effects of minerals on antibacterial metabolite production was evaluated by addition of $MgCl_2$, $CuSO_4$, $MnSO_4$, $FeCl_3$, $CoCl_2$, K_2HPO_4 and $ZnCl_2$, each at a concentration of 0.05% (w/v) in the optimized medium with superior carbon and nitrogen sources.

Results and Discussion

Rsk4 was halophilic, reddish orange colored, gram-positive, aerobic, catalase-positive, non-encapsulated coccoid and non-endosperm forming bacteria. The 16S rDNA sequence (1361 bp) of the strain rsk4 was determined and submitted to GenBank under the accession number HQ258887. On the basis of morphological, physiological, biochemical and analysis of the 16S rDNA sequence, the strain rsk4 was designated as *Kocuria* sp. strain rsk4.

Antibacterial activity of strain rsk4 was detected on starch casein (SC) medium using agar well diffusion method against several pathogenic gram-positive and gram-negative bacteria (Fig. 1). Antimicrobial activity of *Kocuria* sp. was recently reported by Palomo et al. (20) and Ballav et al. (21). It was showing broad spectrum activity and highest antagonistic effect against multiple drug resistance bacteria *S. aureus*. The similar antibacterial activity against *S. aureus* has been reported in halotolerant alkaiphilic *Streptomyces* EWC 7(2) and *Streptomyces* strain CH54-4 by Vijay et al. (22) and Rattanaporn et al. (23). *S. aureus* is multiple

drug resistance human pathogens which can cause various infections. Pediatric patients are frequently affected by these infections and healing options are also limited (24). Therefore, antibacterial metabolites isolated from rsk4 strain could be an important therapeutic against *S. aureus*.

***Optimization for production of Antibacterial compound*:** Optimization of antibiotic production requires complete knowledge on optimal fermentation conditions (25). In the present study, all required conditions and media components had been optimized for the production of antibacterial compound using strain rsk4.

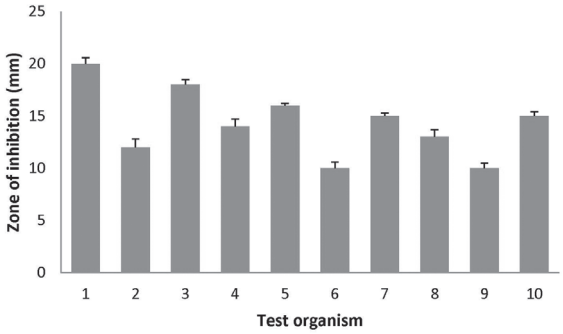

Fig. 1 Production of antibacterial metabolite by rsk4 strain against test organisms such as 1, *Staphylococcus aureus*; 2, *Mycobacterium smegmatis*; 3, *Bacillus subtilis*; 4, *Bacillus megaterium*; 5, *Enterococcus faecalis*; 6, *Klebsiella pneumoniae*; 7, *Proteus vulgaris*; 8, *Salmonella typhimurium*; 9, *Pseudomonas aeruginosa* and 10, *Escherichia coli*.

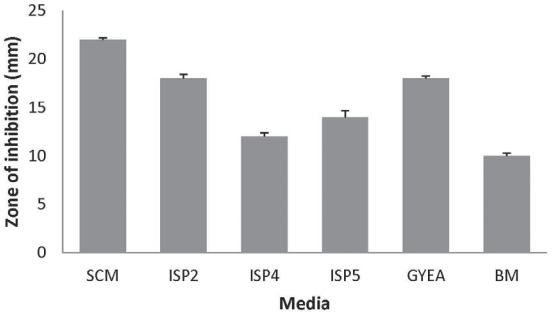

Fig. 2 Effect of different media on antibacterial metabolite production

Effect of media: All media are found to be suitable for antibacterial metabolite production by strain rsk4 but amount of zone of inhibition was varied. Among the six selected media SC medium showed good antibacterial activity against *S. aureus* (Fig. 2). Variation in the antibacterial metabolite production among media could possibly be related to the composition of the medium in which the strain was grown. SC contains three nitrogen sources which might be playing important role in metabolite production. Similar effect of improved antimicrobial metabolite production in starch casein media was shown by marine actinomycetes *Streptomyces afghaniensis* (26).

Effect of incubation periods: The antibacterial metabolite production by rsk4 was monitored over a period of 10 days. The bioactive metabolite production started from 5th day and reached optimum on 7th day (22 mm) but decreased gradually from 8th day. Growth patterns of strain showed that trophophase to idiophase shift occurs during 5th to 6th day (Fig. 3). Antibacterial metabolites production started during stationary phase that confirmed the compound to be a secondary metabolite. Conditions for secondary metabolite production are more restricted than the growth conditions, thus the efficient conversion from the trophophase to the idiophase is important for the production of antibiotics (27). The active metabolic product was not stable and decreases with respect to increasing incubation time. Similarly Jose et al. (28) optimized the required time courses for antibiotic production which was suppressed with the increase in incubation period in the production medium.

Effect of Temperature and agitation: Temperature has impressive effect on the physiology, biochemistry and metabolites production of organisms. It was observed that the culture filtrate of rsk4 had highest antagonistic effect in the range of 25ÚC-30ÚC shown in Fig. 4. There was no antibacterial activity after 40ÚC which states that deviation from optimum temperature affects the efficiency of metabolite. These results accord with a fact, that extreme

temperature are not suitable for antibiotic production (29).

Agitation speed affect oxygen supply and increases contact time of organism with media components. Fig. 5 shows significant differences of metabolite production in different range of agitation. The optimum antibacterial compound production was observed at 180 rpm. Production of antibacterial compound is decreases gradually with increasing rotary speed beyond 180 rpm. This result shows similarity with Bhavana et al. (30) which showed maximum antibiotic activity at 180 rpm and decreases gradually with increasing speed.

***Effect of Salinity and pH*:** Salt concentration effects on the osmotic pressure to the medium and hence marine organisms respond to different salinity conditions to produce secondary metabolites. Rsk4 is halophilic actinomycetes and able to grow at high salt concentration up to 15%, but produces maximum antibacterial compound at 5% NaCl (Fig. 6). Low salinity may effectively create stressful environment condition for halophilic strain, and a response to such stress may include alterations in the secondary metabolite spectrum (31).

pH affect cellular developments such as regulation of the biosynthesis of microbial bioactive metabolites (32) and hence change in pH of the

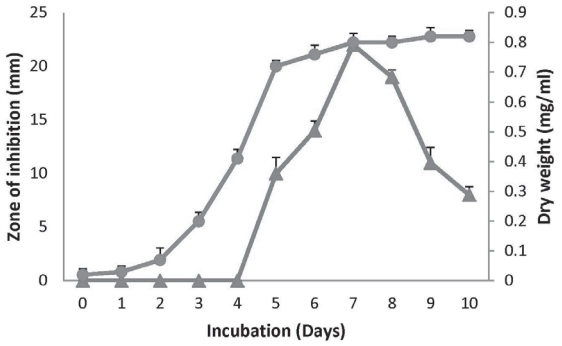

Fig. 3 Effect of incubation period on growth (circles) and antibacterial metabolite production (triangles)

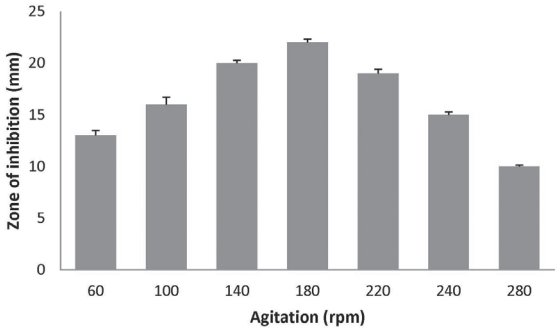

Fig. 5 Effect of agitation on production of antibacterial metabolite

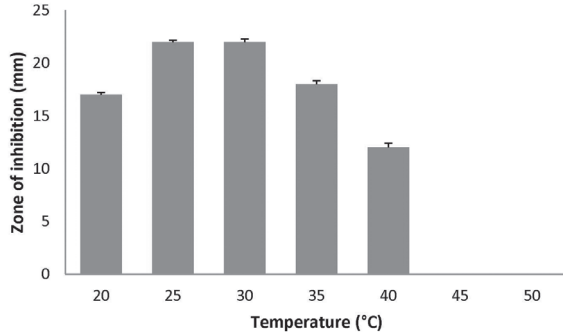

Fig. 4 Effect of temperature on production of antibacterial metabolite

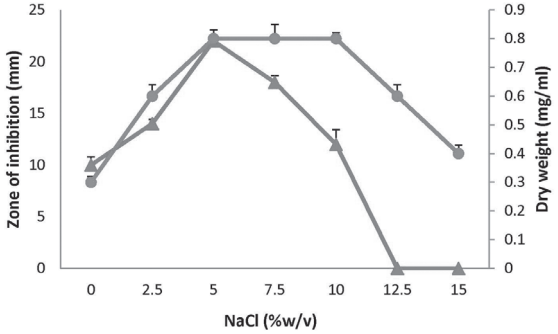

Fig. 6 Effect of salinity on production of antibacterial metabolite

culture medium induces the production of new products that adversely affect antibiotic production. Present study revealed that the optimal pH for production of antibacterial compound by rsk4 strain is neutral pH (Fig. 7). Similar report was shown by Vijay et al. for maximum antimicrobial metabolite production at pH 7 by *Nocardiopsis* sp (33).

***Effect of Carbon and Nitrogen sources*:** Addition of carbon and nitrogen sources in the production media optimally effects the production of antimicrobial compounds (34). Starch was found to be most suitable carbon source for production of antibacterial metabolites by rsk4 shown in Fig. 8. Various studies suggest that the continuous and gradual hydrolysis of starch can avoid inhibition in the production of antibiotics that are normally triggered by simple sources and more easily metabolized by the microorganism (35-36). Among the variable concentration of starch, 1% starch exert more effect on antibacterial metabolite production (Fig. 9).

The nature and concentration of nitrogen sources are considered as direct precursors for the synthesis of antibacterial compound. The effect of various nitrogen sources on antibacterial metabolites production by rsk4 is shown in Fig. 10. Organic nitrogen sources produced better amount of antimicrobial metabolites compared to inorganic nitrogen. The highest activity was obtained with both casein and yeast extract

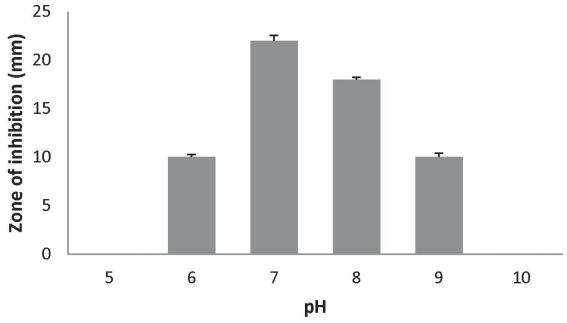

Fig. 7 Effect of pH on production of antibacterial metabolite

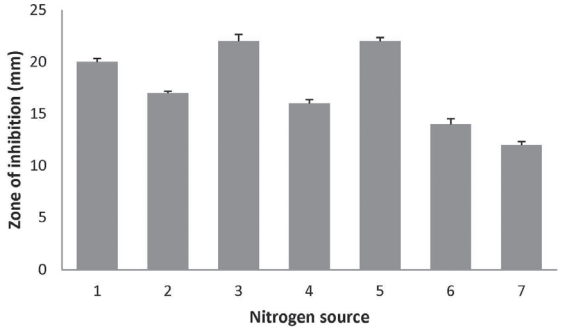

Fig. 9 Effect of various concentration of starch on production of antibacterial metabolite

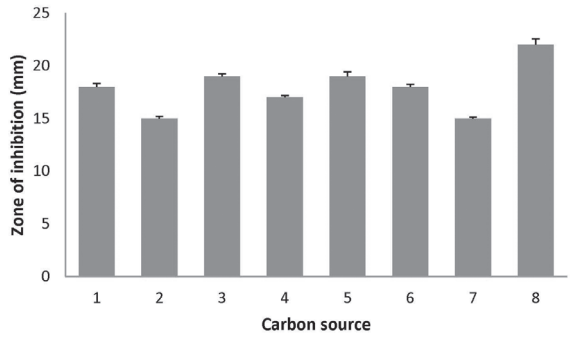

Fig. 8 Effect of carbon source on production of antibacterial metabolite 1, Dextrose; 2, Glucose; 3, Sucrose; 4, Maltose; 5, Mannitol; 6, Lactose; 7, Glycerol; 8, Starch

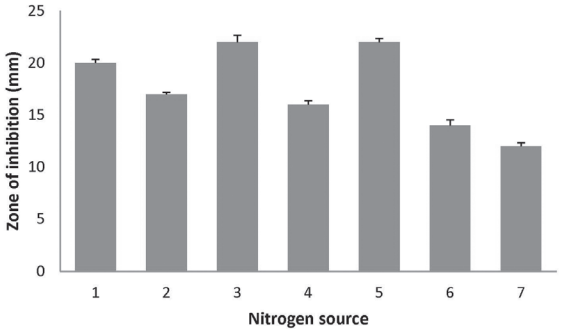

Fig. 10 Effect of nitrogen source on production of antibacterial metabolite 1, Peptone; 2, Beef extract; 3, Yeast extract; 4, Malt extract; 5, Casein; 6, Ammonium sulphate; 7, potassium nitrate

followed by peptone. This study suggests that antibacterial metabolite production is inhibited by a rapidly utilized nitrogen source. Among the various combination of organic nitrogenous source studied, casein + yeast extract showed maximum yield of antibacterial metabolites.

***Effect of Minerals*:** The effect of minerals is represented in Figure 11 which suggests that $MgCl_2$ exert best impact on production of antibacterial metabolites by strain rsk4. $CuSO_4$ did not show any increased metabolite production. This result shows similarity with Sujatha et al. where addition of 0.5 g/l of magnesium sulfate to the culture medium was optimal for antibiotic production by *Streptomyces sp* (37).

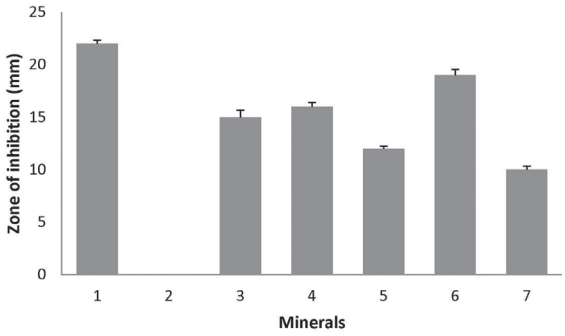

Fig. 11 Effect of minerals on production of antibacterial metabolite 1, $MgCl_2$; 2, $CuSO_4$; 3, $MnSO_4$; 4, $FeCl_3$; 5, $CoCl_2$; 6, K_2HPO_4; 7, $ZnCl_2$

Conclusion

Present investigation involved antibacterial activity of halophilic actinomycetes *Kocuria* sp. strain rsk4 and optimization of culture conditions for maximal metabolite production. Strain rsk4 showed broad spectrum activity and highest antagonistic effect against multi drug resistance *S. aureus*. It exhibited optimum antibacterial metabolite production in starch casein medium at 30ºC temperature, pH 7 and salinity 5% with agitation rate 180 rpm. Optimization of media components suggest that starch, yeast extract, casein and magnesium can increase the production of antibacterial metabolite. This study proves that non-statistical one factor at a time method is efficient, relatively simple and cost effective method and it can significantly increased antibacterial metabolite production.

Table 1 Effect of different combination of nitrogen source on production of antibacterial metabolite

Source	Zone of inhibition (mm)
Peptone + Yeast extract	21.5 ± 1.4
Peptone + Casein	21 ± 1.72
Peptone + Beef extract	18.2 ± 1.4
Peptone + Malt extract	17.3 ± 1.6
Yeast extract + Beef extract	20.2 ± 1.9
Yeast extract + Malt extract	18.5 ± 1.1
Casein + Yeast extract	25.2 ± 0.9
Casein + Beef extract	20.3 ± 1.1
Casein + Malt extract	20.4 ± 1.4
Beef extract + Malt extract	15.3 ± 0.9

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Chemometric-assisted UV Spectrophotometric and RP-HPLC Methods for the Simultaneous Determination of Caffeine and Sodium Benzoate in Synthetic Mixture

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Abstract

In present work, chemometric-assisted UV spectrophotometric and RP-HPLC methods weredeveloped for the simultaneous estimation of caffeine (CAF) and Sodium benzoate (SB) in their combined pharmaceutical dosage form. The two chemometric methods i.e. principle component regression (PCR) and partial least square regression (PLS) were successfully applied to quantify each drug in mixture using UV absorption spectra in range of 220 – 280 nm at $\Delta\lambda$ of 1 nm. Chemometric model development was done using 30 binary solutions and 10 solutions were used for validation of models. The chemometricmethod does not require any prior separation step. In addition RP-HPLC method was developed using HiQSil C18 column with a mobile phase consisting of methanol: acetate buffer (50:50 % v/v), pH adjusted to 4.4 with UV detection at 224 nm and flow rate of 1 ml/min. The methods were successfully applied for the simultaneous determination of these drugs in synthetic mixture.The results obtained for analysis by PCR and PLS methods were compared with RP-HPLC method and a good agreement was found.

Keywords: Chemometric, PCR, PLS, HPLC, Caffeine, Sodium benzoate

Introduction

Caffeine (CAF) is chemically1, 3, 7-trimethylpurine-2; 6-dione(Figure 1a)and is a well-known CNS stimulant.CAFstimulates medullary, vagal, vasomotor and respiratory centers, promoting bradycardia, vasoconstriction and increased respiratory rate by antagonists at adenosine-receptors within the plasma membrane of virtually every cell (1). Sodium benzoate (SB) is added to increase the solubility of CAF and its structure is depicted in Figure 1b. The USP specifies gravimetric assay for CAF and titrimetric method for SB (2).

Chemometric was introduced in 1972 by SvanteWold (3). Chemometric is the science of extracting information from chemical systems. Multivariate calibration method (e.g., multiple linear regression (MLR), principle component regression (PCR) and partial least squares (PLS) utilizing spectrophotometric data are the important chemometric approach for determination of mixtures including drugs combination (4).

Literature survey reveals that there are reported methods on estimation of CAF and SB in combination by UV (5, 6) and HPLC(7) methods. The reported UV spectrophotometric methods are based on multicomponent analytical

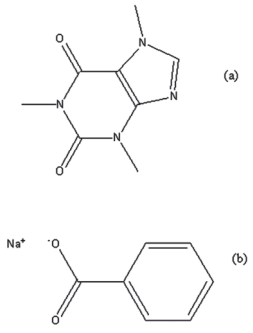

Fig. 1. Chemical structure of (a) caffeine and (b) sodium benzoate

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method butin the present work, the chemometricmultivariate calibration methods are applied for the multicomponent analysis of drug substances which provides more powerful analysis tool. HPLC method was also developed and validated for the simultaneous determination of CAF and SB. The injection sample (blend)was assayed with the optimized chemometric-assisted spectrophotometric method and HPLC method for comparison.

Material and Methods

Instrumentation: The apparatus used for chemometric analysis was double beam UV-Vis spectrophotometer (Jasco V-550, Japan) with matching pair of 1 cm quartz cells. The absorbance spectra of calibration set and validation set were recorded in range of 220-280 nm with $\Delta\lambda$ of 1 nm. PCR and PLS calculations were performed on Unscrambler X 10.3 (trial version) and MS-Excel 2007.

The RP-HPLC was carried on JASCO HPLC (PU 2080 Plus, Japan) equipped withJasco PDA detector (PU 2010 Plus, Japan). Samples were injected through Rheodyne sample injection port (20 µl). HiQSilC18 Column(250 x 4.5mm, i.d. 5 ìm) was used. Data acquisition and integration was performed usingBorwin software (version 1.5).

Material and Reagent: Pure drug samples of Caffeine and Sodium benzoate were kindly gifted by S. D. Fine Chemicals ltd. (Mumbai, MH, India). The standards were used without further purification. HPLC grade water was obtained from ELGA LABWATERpurification system (PURELAB UHQ-11, United Kingdom). Methanol used for HPLC was of HPLC grade and that used for spectrophotometry was of AR grade (LOBA Chemie, Mumbai, MH, India).

Chromatographic Conditions: The mobile phase was prepared by mixing methanol and 10mMAcetate buffer (pH adjusted to 4.4 using glacial acetic acid) in ratio (50:50 % v/v). The flow rate was 1 ml/min. Quantitation based on peak area was achieved using PDAdetector at 224 nm. All determinations were performed at ambient temperature.

Standard stock solutions: Stock solution of CAF and SB were prepared by dissolving accurately weighed 10 mg of standard drugs in 10 ml of double distilled water, separately (1000 ìg/ml). Gentle warming was carried out to dissolve caffeine properly in water. From above solution further 5 ml was pipetted and diluted to 50 ml (100 ìg/ml) of CAF and SB, respectively.

Working solutions: Working standard solutions were prepared from standard stock solution of 100 µg/ml by appropriate dilution to obtain final concentration of 2.5-15 µg/ml for HPLC (dilution with mobile phase) and 5-25 µg/ml for chemometric analysis (dilution with double distilled water) for both the drugs.

Construction of calibration and validation set for chemometric approach: A total set of 40 mixtures were prepared by combining working standard of CAF and SB in their linear concentration range of 5-25 µg/ml for both drugs (Table 1). From these 30 mixtures were used for calibration set and 10 mixtures were used for validation set. The validation set was randomly selected. The absorbance spectra were recorded in range of 220- 280 nm with 1 nm interval.The absorbance data of the calibration set were then processed throughUnscramblersoftware for development of PCR and PLS models. For validation generated models, the concentrations of drugs in validation set were predicted by using the proposed PCR and PLS models.

Single Component Calibration: To find linear concentration range of each drug, one component calibration was performed. Linear dynamic ranges were studied in the concentration range of 5.0-30.0 µg/ml for both drugs. Figure 2 represents overlain spectra CAF and SB.

Calibration curves for the HPLC method: Working solutions in the concentration range of 2.5-15.0 µg/ml were injected in triplicateand chromatogram was obtained under the specified chromatographic conditions described previously. The calibration graph was constructed by plotting peak area versus concentration of each drug and the regression equation was calculated.

Analysis of Drug in synthetic mixture: The sample solution of injection of caffeine and sodium benzoate was prepared in the laboratory as it was not easily available in the market. The injection was prepared by dissolving caffeine in water for injection with sodium benzoate to increase the solubility of caffeine. The volume was made up to make a solution containing dose of 100 ìg/ml each. The sample solution of injection was then used for assay performance and % recovery studies by preparing dilutions in double distilled water to get standard concentration of 10µg/ml each, procedure was repeated for six times

Validation by HPLC method: For validation of the developed method, the ICH Q2 (R1) guidelines were followed. The requirement for drug assay follows these topics: linearity, precision, accuracy, specificity, robustness, LOD and LOQ.

Linearity*:* Working standard solution of the drug was diluted to prepare linearity standard solutions in the concentration range of 2.5-15 µg/ml of CAF and SB, respectively. Six sets of such solutions

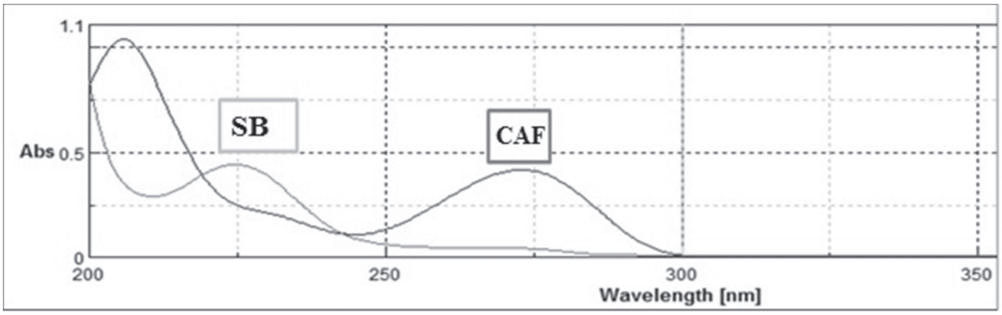

Fig. 2. Overlaid spectra of caffeine and sodium benzoate(10 µg/ml each)

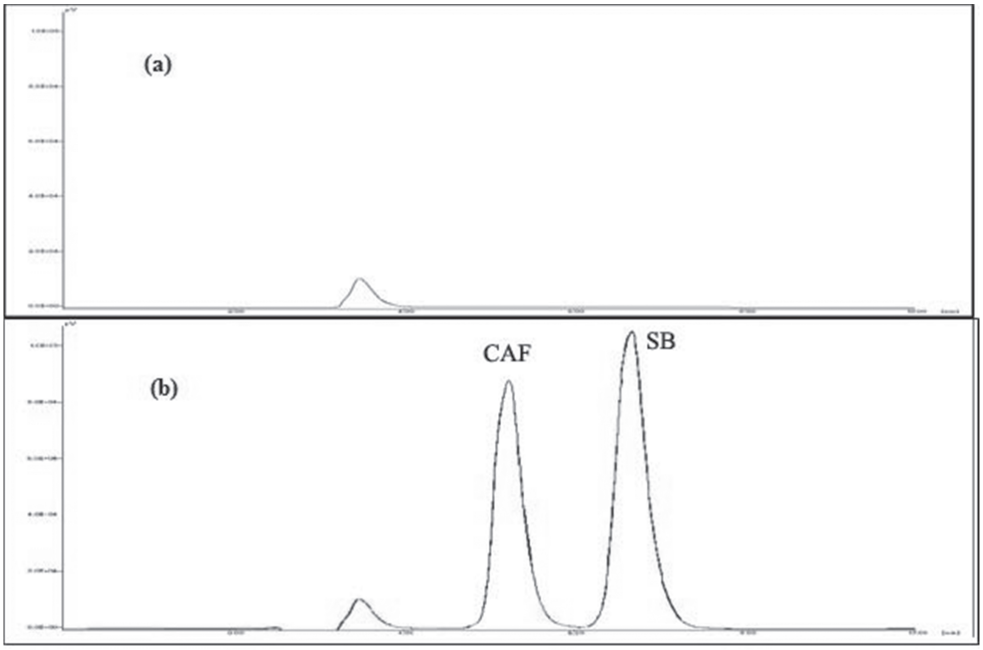

Fig. 3. (a) Blank (b) Chromatogram of caffeine and sodium benzoate

were prepared. Each set was analyzed to plot a calibration curve. Standard deviation (SD), slope, intercept and correlation coefficient (R^2) of the calibration curves were calculated to ascertain the linearity of the method.

Precision: The precision of the method was demonstrated by intra-day and inter-day variation studies. In the intra-day studies, 6 replicates of CAF (10 µg/ml) and SB (10µg/ml) were analyzed in a day and % RSD was calculated. For the inter day variation studies, 3 different concentrations (5, 7.5 and 10 µg/ml) were analyzed on 3 consecutive daysfor both the drugs and % RSD were calculated.

Accuracy*:* To check accuracy of the method, recovery studies were carried by spiking the standard drug to the sample injection solution, at three different levels of 50, 100 and 150 %.The % recovery and % RSD were calculated.

Specificity*:* The specificity of the method was ascertained by injecting placebo preparations without main drugs in the mixture and further confirmed by peak purity profiling studies. The peak purity values were found to be more than 991, indicating no interference of excipients at analytes Rt.

Robustness*:* Robustness of the method was determined by carrying out the analysis under conditions during which mobile phase composition, detection wavelength, flow rate were altered and the effect on the area were noted.

Limit of Detection (LOD) and Limit of Quantitation (LOQ) : The limit of detection (LOD) and limit of quantitation (LOQ) were separately determined at a signal-to-noise ratio (S/N) of 3 and 10. TheLOD and LOQ were theoretically verified by the equations. LOD = 3.3 σ/Sand LOQ = 10σ/S, where, σ is the standard deviation of the intercept and S is the slope of calibration curve.

Result and Discussion

Chemometric method: The first step in multivariate methods involved constructing the calibration matrix. The wavelength range used was 220 to 280 nm. Sixty one spectral points at 1 nm intervals were selected within this range. The compositions of the calibration mixtures were randomly designed in order to collect maximum information from the spectra of these mixtures.The quality of multicomponent analysis is dependent on the wavelength range and spectral mode used. The PCR and PLS models were developed by the Unscrambler® program. Model development was performed by using 30 mixtures as calibration standards. Leave-one-out cross-validation (LOO-CV) was used to validate the PCR and PLS models in model development and obtain optimum latent variables (number of factors) of the model. The predicted concentrations of the components in each sample were compared with the actual concentrations and the root mean square error of cross validation (RMSECV) was calculated for each method. The RMSECV was used as a diagnostic test for examining the error in the predicted concentrations. The resulting models were also validated by prediction of the concentration of analytes in a separate validation set shown in Table 2. Statistical parameters of the chemometric methods are represented in Table 3.

HPLC method: The validity of the analytical procedure as well as the resolution between the peaks of interest is ensured by the system suitability test (Table 4). All critical parameters tested met the acceptance criteria. As shown in the chromatogram (Figure 3), the two analytes are eluted by forming symmetrical single peaks well-separated from each other. CAF and SB showed a good correlation coefficient in the concentration range of 2.5-15 µg/ml. The linear regression analysis obtained by plotting the peak areas of analytes vs. concentration showed excellent correlation coefficients (correlation coefficients greater than 0.990). The proposed method afforded high recoveries of almost 100 % for CAF and SBin synthetic formulation, indicating that this assay procedure can be used for the routine quality control analysis of the pharmaceutical dosage form.

The system precision is a measure of the method variability that can be expected for a given

Table 1. Calibration set and Validation set

Mix. No	SB	CAF	Mix. No	SB	CAF
1	5	5	21	25	5
2	5	10	22	25	10
3	5	15	23	25	15
4	5	20	24	25	20
5	5	25	25	25	25
6	10	5	26	5	30
7	10	10	27	10	30
8	10	15	28	15	30
9	10	20	29	20	30
10	10	25	30	25	30
11	15	5	31	10	5
12	15	10	32	15	5
13	15	15	33	20	10
14	15	20	34	25	10
15	15	25	35	5	15
16	20	5	36	10	15
17	20	10	37	10	20
18	20	15	38	30	20
19	20	20	39	15	25
20	20	25	40	30	25

1-30: Calibration set; 31-40: Validation set

Table 2. Percentage recovery results of caffeine and sodium benzoate in validation set by the proposed PCR and PLS chemometric methods.

METHOD		PCR				PLS			
SB	CAF	SB		CAF		SB		CAF	
Actual (ìg/ml)		Predicted	% R*	Predicted	% R*	Predicted	% R*	Predicted	% R*
10	5	9.928	99.2	5.041	100.8	9.963	99.6	5.144	102.8
15	5	15.517	103.4	5.123	102.4	15.125	100.8	5.138	102.7
20	10	20.371	101.8	10.085	100.8	20.312	101.5	10.051	100.5
25	10	24.830	99.3	9.988	99.8	24.891	99.5	9.987	99.8
5	15	4.960	99.2	14.915	99.4	4.971	99.4	14.892	99.2
10	15	10.126	101.2	14.948	99.6	10.029	100.2	14.970	99.8
10	20	10.013	100.1	20.161	100.8	10.113	101.1	19.866	99.3
30	20	29.816	99.3	20.132	100.6	29.768	99.2	20.193	100.9
15	25	15.017	100.1	24.941	99.7	15.087	100.5	24.913	99.6
30	25	29.774	99.2	25.024	100.0	29.757	99.1	25.128	100.5

%R*: percent recovery

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Table 3. Statistical parameters of caffeine and sodium benzoate using the proposed PCR and PLSchemometric methods

Statistical Parameters	SB		CAF	
	PCR	PLS	PCR	PLS
Range (ig/ml)	5.0-30.0		5.0-30.0	
Offset	0.039	0.039	0.029	0.029
Regression Coefficient (R^2)	0.995	0.995	0.997	0.997
RMSE	0.714	0.714	0.616	0.616
PC/Factors	2		2	

Table 4. System suitability parameters for CAF and SB

Drug	Concentration (ig/ml)	RT (Min)	Area	Plates	Asymmetry	Resolution
CAF	10	5.30 ± 0.05	772011	4546	1.04	2.73
SB	10	6.90 ± 0.09	2171667	3897	0.98	3.12

Table 5. Summary of validation parameters by proposed HPLC method

Sr. No.	Validation Parameter	CAF	SB
1.	Regression Equation	y = 93883x + 17560r^2 = 0.998	y = 19060x + 27869 r^2 = 0.992
2.	Range	2.5-15 µg/ml	2.5-15 µg/ml
3.	Intraday precision (% RSD)	0.505	1.337
4.	Interday precision (% RSD)	1.427	1.584
5.	LOD (µg/ml)	0.419	0.464
6.	LOQ (µg/ml)	1.270	1.407
7.	Accuracy (% recovery)	100.150	100.500
8.	Robustness (% RSD)	<2	<2
9.	Specificity	Specific	Specific

Table 6. Assay results of CAF and SB by the proposed PCR, PLS chemometric methods and HPLC method

DRUG Actual amount	**CAF** 5µg/ml			**SB** 5 µg/ml		
Method	HPLC	PCR	PLS	HPLC	PCR	PLS
*Amount found	4.984	4.998	4.979	5.029	5.007	5.009
% Amount found	99.68	99.97	99.59	100.58	100.15	100.18
P value		0.7983			0.7148	
F value		0.2287			0.3435	

*Average of 6 determinations for HPLC, PCR and PLS methods.

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analyst performing the analysis and was determined by performing six repeats. The %RSD for CAF and SB response was found to be less than 2.0 (values in limit). The intermediate precision was assessed by analyzing three different concentrations from the calibration linearity on three different days and intraday precision was assessed by analyzing three different concentrations from the calibration curve on the same day. % RSD for precision was in limit. The summary of HPLC results are depicted in Table 5.

Comparison of the Chemometric method with the HPLC Method: In order to compare the results of the proposed PCR and PLS models for the determination of CAF and SB in synthetic mixture, the HPLC method was also employed. The same samples solutions used for the PCR and PLS models were analyzed by the HPLC method. The determination results of PCR, PLS, and HPLC methods are presented in Table 6. The data were expressed in terms of percent labeled amount. The results showed that the average percent labeled amount obtained from the PCR and PLS models were not significantly different from those obtained from the HPLC method with the confidence limit of 95%.

Conclusion

RP-HPLC techniques are generally used for separation and determination of components in pharmaceutical formulations and are considered superior with regard to identification and specificity. However, the chemometric methods are less expensive by comparison and do not require sophisticated instrumentation nor any prior separation step. The proposed chemometric-assisted spectrophotometric methods are applicable and specific for the simultaneous determination of CAF and SB in their synthetic mixtures. The results obtained were compared with the proposed RP-HPLC method and good coincidence in the means of recovery was observed as there was no significant difference between the methods compared. The three proposed methods were accurate, precise with good reproducibility and sensitivity; hence can be used for the routine analysis of these in their combined pharmaceuticals.

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Identification of Quantitative Trait Loci for Panicle Associated Traits in Recombinant Inbred Line Population Derived from *Japonica X Indica* sub-species in Rice (*Oryza sativa* L.)

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Abstract

Rice recombinant inbred lines (RILs) of *Japonica* x *Indica* cross were grown in irrigated conditions for identification of quantitative trait loci (QTLs) for panicle associated traits. One hundred twenty one RIL lines were evaluated in the field environment with two replications. A total of one hundred twenty two SSR markers were used for polymorphism out of forty markers that showed parental polymorphism. Forty SSR markers were used to construct the genetic linkage map by employing single marker analysis. Maximum number of QTLs was identified for total number of spikelets per panicle and minimum for panicle weight per plant. Clustering of QTL for different traits at the same marker RM 224 and RM 312 was observed for total number of spikelets per panicle, filled spikelets per panicle and panicle weight per plant. This suggests that pleiotropism and or tight linkage of different traits could be possible for the congruence of several QTLs. Moreover, phenotypically these characters have more association with each other. Hence, these markers may be useful for marker assisted breeding programme.

Key words: Rice, Single marker analysis, Panicle traits, QTL identification.

Introduction

Rice is a staple food for more than half of the world's population including two billion Asians, who obtain 60-70% of their energy intake from rice and its derivatives. Rice is globally grown on about 154 million hectares annually with total production of 600 million tons. To meet the growing demand from human population which is expected to touch 9 billion by 2050, in a changing global climatic order, rice varieties with higher yield potential and greater yield stability need to be developed. One of the means of achieving the projected production demand is by integrating classical breeding techniques with modern biotechnological tools for rice improvement (1). In rice panicle length, grains per panicle and grain sterility are crucial determinants of grain yield together with number of panicles per plant (2). These traits are inherited in a quantitative manner and typically controlled by a plurality of major and minor QTLs. A key development in the field of complex trait analysis was the discovery of DNA based genetic markers, physical establishment of high density genetic maps and development of QTL mapping methodologies such as single marker analysis, interval mapping and multitrait mapping.

There are number of reports on mapping and introgression of QTLs from wild species in rice. However, the related subspecies of *Oryza sativa* such as *japonica* carry many useful alleles which can be used for improvement of *Indica*. It has been observed that derivatives of *indica/japonica* cross have higher yield vigour than either *indica/ indica* or *japonica/japonica* derivatives. Therefore,

identifying the chromosomal locations influencing yield and yield related traits in inter subspecific derivatives is useful for rice improvement. Identification of favourable alleles in *japonica/indica* will pave way to the marker assisted mobilization of their allele in a genetic background to break genetic barrier for higher yields. The objectives of the present study were to identify QTLs and map genomic regions influencing panicle associated traits using phenotyping data from a recombinant inbred population generated by inter subspecific cross between JNPT 89 (*Japonica x Indica*) with IR 64 which is highly adopted and a high yielding *indica* rice variety.

Materials and Methods

The material for this study consisted of F_{10} one hundred twenty one Recombinant Inbred lines developed in the cross of JNPT 89 & IR 64. Marker analysis selective genotyping method was used to detect the association of QTLs with panicle associated traits. The RILs along with parents were planted in an Alpha lattice design with two replications at Seed Breeding Farm, Department of Plant Breeding and Genetics, J.N.K.V.V., Jabalpur. Twenty-one-day seedlings of each genotype were planted in five rows of three meter length with 20 cm row spacing keeping single seedling per hill. Recommended package of practices were followed to raise a good crop. Observations were recorded on randomly selected five plants from each genotype, in each replication at maturity. These plants were harvested and thrashed separately. The data were recorded for panicle traits viz., panicle length, number of filled grains per panicle, total number of spikelets per panicle and panicle weight per plant and grain yield per plant.

DNA isolation *:* Preparation of genomic DNA from the parents and RILs followed the mini prep method. The extracted DNA content was quantified and parental polymorphism studies were carried out through 112 SSR primers. PCR mix for one reaction (volume 20 µl) contained 2 µl DNA, sterile and nanopure water 13.5 µl, 10x assay buffer, 1 µl dNTP, 0.5 µl of each forward and reverse primers, and 0.5 µl Taq DNA polymerase. PCR amplification was performed with the following steps: pre denaturing at 94! for 4 min, followed by 35 cycles of 94! for 1 min, 55! for 1 min and 72! for 2 min, and last step for 5 min at 72!. Amplified products were analysed using 5% polyacrylamide gel. Electrophores was carried out for 1 hr at 199 volts. The gel along with the DNA sample was stained with eithidium bromide (10 µg/10 ml) for 40-45 min. Gel was visualized on UV-transilluminator and image was observed on the computer screen.

SSR assay and linkage analysis :For the SSR assay, 40 SSR primer pairs of 112 microsatellite markers (SSRs) derived from Cornell SSR linkage map (3) was tested on JNPT 89 and IR64, which showed polymorphism between the parental DNAs. A total of 40 SSR primer pairs were analyzed for the population. Test for QTL association with traits was performed by single marker approach. The single marker analysis, t-test was followed to find out the significant association between traits and the markers. Single marker analysis, t-test was calculated for each of the phenotypic traits with all the marker classes. The potential relationship between the marker and trait was established considering the significance of the t-test. It was found that a single marker was related with many traits and a single trait related to many markers.

Data Scoring *:* The female parent band was scored as 'A' while male parent band was scored as 'B', the bands of individual RIL lines were scored either as A or B depending on its position like female and male parent, respectively. The bands other than A and B were termed as E. Test for QTL association with traits was performed by single marker approach. The single marker analysis, t-test was followed to find out the significant association between traits and the markers.

Results and Discussion

The performance of the RILs lines and their parents JNPT 89 and IR 64 in irrigated condition for panicle associated traits is tabulated in table 1. Analysis of variance revealed significant differences ($P < 0.01$) between the two parental

lines in all panicle related traits assessed in the current study. Therefore, it could be expected that the RILs population derived from the cross between the two parents would be suitable for mapping of the QTLs for panicle traits. An approximate normal distribution was observed for phenotypic performance of the traits. A wide variation in the performance of the RIL lines for all traits was observed. However, the performance of parents varied considerably for number of filled grains per panicle, total number of spikelets per panicle and panicle weight per plant while the magnitude of the variation was less in panicle length and grain yield per plant. Maximum variation was observed for total number of spikelets per panicle, while panicle length had the least.

Phenotypic correlation *:* Phenotypic correlations were conducted among the evaluated panicle related traits based on means. The traits with the highest positive significant correlations were found between number of filled spikelets per panicle and number of spikelets per panicle (0.8802) (Table-2).

SSR polymorphisms *:* Polymorphism is recognized as a measurement for genetic diversities between the breeding parents. In this study, a total of 112 SSR markers were used to detect the polymorphism between the parents with 40 SSR markers that showed polymorphism (35.7%). The results show that the rate of polymorphism is lower than generated in the interspecific and inter subspecific crosses, where the polymorphism ranged from 59.6%-90% as reported in some previous studies (4,5,6). The reason for the low polymorphism might be explained that the parents used in this study have higher genetic similarities. The selected 40 polymorphic SSR markers were employed to genotype the F_{10} RIL population (Fig. 1 and Fig. 2).

Table 1. List of RILs showing transgressive segregants for panicle associated traits

Trait	Transgressive Segregants				Parental Value	
	Highest		Lowest			
	RIL Number	Value	RIL Number	Value	JNPT 89	IR64
PL	RIL99	29.55	RIL60	21.20	28.30	25.25
FSN	RIL81	280.30	RIL31	94.05	301.50	126.95
TNS	RIL91	413.40	RIL101	128.61	395.00	135.10
PWPP	RIL91	49.75	RIL102	17.60	31.60	37.10
GYPP	RIL77	47.00	RIL3	9.50	34.85	31.12

Table 2. Estimation correlation coefficient between panicle associated traits in RILs

Character	PL	FSN	TNS	PWPP	GYPP
PL	**1.0000**	0.2049**	0.1693*	0.0804	0.0098
FSN		**1.0000**	0.8544**	0.4793**	-0.0691
TNS			**1.0000**	0.5968**	-0.1661*
PWPP				**1.0000**	0.0720
GYPP					**1.0000**

PL-Panicle Length, FSN- Filled Spikelets Number Per Panicle, TNS-Total Number of Spikelets Per Panicle, PWPP-Panicle Weight Per Plant, GYPP- Grain Yield Per Plant.

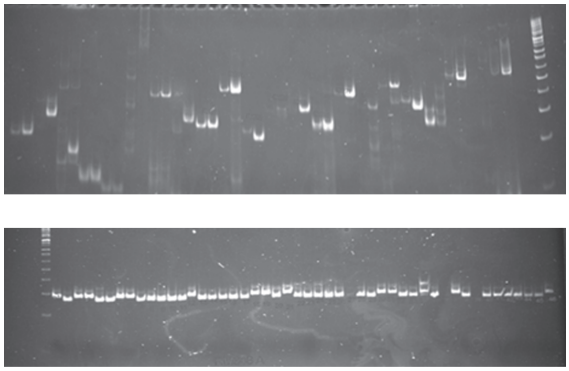

Fig. 1. Gel picture of parental polymorphism

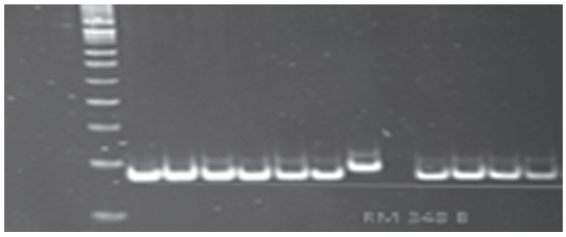

Fig 2. Gel picture of RM 348 used in RIL population

Construction of framework map using SSR markers *:* A total of forty four polymorphic SSR markers evenly distributed on the 12 chromosomes were used for construction of the linkage map with the RIL population. Map order was in agreement with that provided by McCouch *et al.* (2002). Segregation distortion in the population was tested by the X^2 statistics. Such distorted segregations in mapping populations have been frequently reported earlier (7,8).

Association of molecular markers with Panicle traits and QTL analysis : While maximum number of QTLs were identified for total number of spikelets (24), minimum were observed for panicle weight per plant (3) and none of the QTLs were identified for panicle length and grain yield per plants. The QTLs were identified on all the twelve chromosomes presented in Table 3 and Fig 3. Filled spikelets per panicle exhibited association with twenty markers viz., RM348, RM341, RM231, RM248, RM279, RM312, RM16, RM337, RM495, RM517, RM552, RM206, RM212, RM269, RM190, RM164, RM208, RM247, RM474 and RM250. All twelve chromosomes except chromosome number 6 showed association for filled spikelets per panicle (4, 5, 6, 9, 10). All chromosomes contained QTLs for total number of spikelets per panicle. Total number of spikelets per panicle showed association with RM235, RM348, RM341, RM231, RM248, RM224, RM312, RM16, RM337, RM495, RM206, RM212, RM269, RM204, RM553, RM221, RM190, RM164, RM208, RM247, RM474 and RM250. This result is in agreement with the finding of QTLs for total number spikelets per panicle on chromosome 1, 6 and 9 (11) and on chromosome 1, 2, 3, 9 and 12 by using an advanced backcross population between *Oryza rufipogon* (IRGC 105491) x Jefferson (6).

Panicle weight per plant had association with RM 312, RM 553 and RM 224 located on chromosome 1, 9 and 11 at locus 71.6 cM, 76.7 cM and 120.1 cM respectively. Pursuing high grain

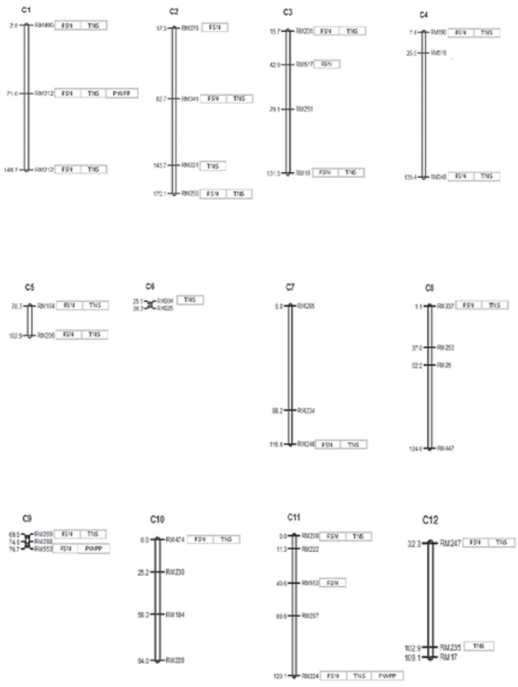

Fig. 3. Molecular linkage map showing the position of filled spikelets per panicle (FSN), total number of spikelets per panicle (TNS) and panicle weight per plant (PWPP) QTLs identified in RIL population.

Table 3. Single marker analysis (t-test) of SSR primer for RILs

S.No.	Markers	PL	FSN	TNS	PWPP	GYPP
1	RM235	1.79	1.93	2.06*	-0.28	0.05
2	RM348	0.66	4.86**	3.78**	0.22	-1.92
3	RM341	0.26	3.85**	3.34**	0.89	-0.62
4	RM231	1.04	3.25**	2.66**	1.27	-1.46
5	RM248	-0.55	2.66**	2.74**	1.27	-0.96
6	RM224	0.61	3.30	3.78**	2.92**	-1.48
7	RM279	0.42	2.69**	1.66	0.46	-1.01
8	RM312	1.10	3.55**	3.15**	2.44*	-0.16
9	RM16	-2.63	2.15*	2.13*	0.54	-1.63
10	RM337	0.08	2.17*	3.12**	0.97	-2.42
11	RM495	-0.09	2.86**	2.83**	-0.70	-1.91
12	RM517	-2.12	2.58**	1.87	1.60	-0.94
13	RM552	0.50	2.32*	1.36	0.37	0.05
14	RM206	-0.63	2.19*	2.37*	0.57	-1.17
15	RM212	0.45	4.10**	4.03**	1.92	-0.54
16	RM269	-1.08	2.11*	2.76**	0.27	-1.56
17	RM204	1.07	1.55	2.16*	-1.42	-0.88
18	RM190	1.34	3.88**	3.99**	1.36	-2.89
19	RM553	-0.75	0.91	2.46*	2.85**	-0.74
20	RM221	0.27	1.44	3.07**	1.15	-1.77
21	RM164	-1.89	2.09*	2.74**	0.69	-0.98
22	RM208	-1.05	3.38**	3.33**	0.33	-1.92
23	RM247	-0.37	2.10*	2.13*	-0.51	-1.30
24	RM474	1.56	3.01**	3.41**	-0.48	-3.62
25	RM250	1.28	3.15**	2.61**	0.82	-0.95

* = Significant at 0.05 probability level ** = Significant at 0.01 probability level

yield is one of the most important objectives in rice production. The genetic bases of number of filled spikelets per panicle and total number of spikelets per panicle have received much attention because of their importance in rice breeding. RM 224 and RM 312 showed association with filled spikelets per panicle and total number of spikelets per panicle and panicle weight per plant. The congruence of the QTL loci on the chromosome for various traits may be due to either linkage or pleiotropism. This signifies the plural selection efficiency by selecting markers closely associated with these traits. Since the direction of the additive effect of the QTL was also in the same direction, selection if exerted would be very effective. QTL markers could be fine mapped and made use of for detecting the complex traits like yield and its other contributing traits. In the present study, RIL population should be tested for detecting QTLs across different environments for the stability of QTL for yield and yield attributing traits. Location specific QTLs can be used for selection at specific locations and to develop better genotypes.

In the conclusion, the detection of new QTLs associated with panicle traits should be useful for rice yield improvement in the future. After validation of all tightly linked flanking markers for these QTLs, these markers could potentially be routinely used by breeders for marker assisted selection programs.

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Computational Biology as an aid in Deciphering vital insights on Plant Pathogens and their Interaction with hosts

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Running title – Deciphering Vital Insights on Plant Pathogens and their Interaction

Abstract

Computational biology or bioinformatics has its roots in the dawn of genomic revolution when research activities were started to sequence the genome of various organisms. Refinement in sequencing technologies and potential of genomic research resulted in meteoric growth of biological information such as sequences of DNA, RNA and protein requiring databases for efficient storage, management and retrieval of the biological information. Also, computational algorithms for analysis of these colossal data became a vital aspect of biological sciences. As in other biological sciences, computational biology and genome sequencing gave a new lease of life to plant pathology research, enabling an improved knowledge of the molecular basis of host-pathogen interactions. At present, these genome information of various plant pathogenic fungi, bacteria, virus, phytoplasma etc are stored and managed in various databases. Besides databases for information on nucleotide and proteins sequences of plant pathogens, databases are available for resistance genes for facilitating resistance breeding as well as bioprospecting and allele mining. Analysis of stored information, through bioinformatics tools, can reveal vital information of plant pathogens. Computational algorithms are now an intrinsic part of plant pathology providing valuable solutions in various aspects of plant disease research such as designing diagnostic molecular tools, ascertaining genetic variability among pathogens, taxonomic and phylogenetic studies of plant pathogens, prediction of pathogenicity genes to name a few. Bioinformatics tools give valuable insights into the mechanism of pathogenesis and also help in identifying the underlying strategy involved in the initiation of plant immune response.

Keywords: Bioinformatics, Databases, Genome sequencing, Plant pathology research

Introduction

Food security is paramount issue to humanity as food is the most basic necessity for the survival of each individual. Rapid population growth, 6.07 billion in 2000 to projected 9 billion in 2050 (1), will further pose immense pressure on the food availability among the individuals. According to FAO report, due to deficit in food supply, about 12.5 per cent of the total world population suffers from chronic undernourishment (2), with situations worst in the developing countries.

Various factors stand as hurdles in achieving food security, includes depletion of biodiversity, global water crisis, climate change, land degradation, crop and livestock diseases etc. With no exigency plans in hand, diseases affecting food crops can have devastating effects on food availability. Estimates differ, but about 14.1 per cent of the crop loss occurs due to diseases (3) and jeopardize the current situation of food supply. The causal agents of diseases in crops accounts a vast number of plant pathogens ranging from few nucleotides in viroids to eukaryotic fungi. The effect of the phytopathogens on food crops range from development of mild insignificant

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symptoms to catastrophes, in which vast areas of the crops are ravaged. Epidemics due to plant pathogens can be accounted to Irish famine in 1845, Great Bengal famine in 1942, Southern corn leaf blight epidemic in 1972 etc. In recent times, a lineage of wheat stem rust, Ug99 widely prevalent in the wheat fields across several countries in Africa and the Middle East and anticipated to spread further through these regions and possibly afield, has the potential of causing wheat production desolation with 100% crop loss (4).

Due to diversification in population of plant pathogens in time, latitude and genotype, it is very difficult to formulate control measures. Therefore, to strive the destruction in yield they create, it is necessary to delineate the real cause and explore for the remedies. The classical approaches applied in plant pathology like disease diagnosis by macroscopic symptom observation, microscopic observation of pathogen, transmission test to indicator plants, etc, have been invaluable over the years and will continue to remain so, but the recent advances in molecular and genomic research have revolutionized the approaches. The various processes underlying the interactions of pathogen and host at the molecular level are revealed through advent of molecular biology in plant pathology utilizing various techniques like molecular cloning, polymerase chain reaction, gel electrophoresis, macromolecule blotting and probing, etc. Genesis of genome sequencing further complimented molecular biology enabling a better knowledge at the gene level, of the strategies and underlying mechanisms of pathogenesis of plant pathogens.

The recent explosion in genome sequencing and high throughput sequencing technology has brought about unprecedented quantity of biological information to practically any biological field. With bacteriophage MS2 having RNA genome (5) being the first organism to mark the beginning of genome sequencing which further led to multinational genomic collaborative projects like Human Genome Project (6) and International Rice Genome Sequencing Project (7). In respect of plant pathogens, there are currently complete genome sequences from various plant pathogenic fungi, bacteria and representatives of all plant virus genera are publicly available. This rapid rise in enormous amount of biological data amounting to billions of base pairs has led to an absolute requirement of computerized system for storage, organization and management and efficient retrieval of databases for further analysis. Thus this resulted in emergence of the discipline of bioinformatics or computational biology.

The computational analysis of biological data, *i.e.*, Bioinformatics which includes investigation of the information stored in the form of nucleotide and protein sequences in diverse biological databases has huge application in various fields of sciences including plant pathology. It has worked wonders in the study of plant pathogens, solving problems which otherwise would have been impossible without its use. It not only deals in creation of databases for efficient storage and retrieval but also in development of tools and algorithms for further analysis of stored data.

DNA sequence analysis using Bioinformatics

Ranging from prokaryotic bacteria to multi-cellular higher mammals large amount of sequence data are being published and analysis of this data involves various tasks which includes sequence assembly, annotation, motif finding, sequence alignment and phylogenetic trees (8). Sequencing through shotgun approach in which 1000 base pair long DNA fragments are sequenced with significant over coverage followed by assembling. Annotation follows assembling where genes are identified, marked out and their functions are turned out (9). Non-coding DNA contains regulatory regions where proteins called transcription factors (TFs) bind to turn on genes. TFs typically bind to small motifs, so the task is to find overrepresented short motifs in larger quantities of sequence. In the last two tasks, it is very useful to compare genomes of previously sequenced species. “Comparative genomics” is becoming a very important subfield (10). Detection and alignment of homologous sequence is an important task here. Given sequence data from

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different species, it is useful to reconstruct their phylogenetic relationship. Algorithms exist for all these tasks, but all are evolving with increasing understanding of the function of non-coding DNA, increasing mathematical and algorithmic sophistication in the methods, and increasing raw computational power available to tackle these tasks.

Bioinformatics databases for plant pathology research : Databases, assemblage of structured, systematic and up-to-date information regarding the sequence of the three most important biomolecule *i.e.* DNA, RNA and protein are of the types, the nucleotide databases and the protein databases. The three primary nucleotide databases, which together comprise the International Nucleotide Sequence Database Collaboration are GenBank (National Centre for Biotechnology Information), EMBL (European Molecular Biology Laboratory) and DDJB (DNA Databank of Japan) (11). On the other hand, the leading protein sequence databases are Protein Data Bank (PDB), Universal Pesource (UniProt), Protein Information Resource (PIR) and SwissProt (12). These databases contain nucleotide sequence of thousands of organisms which include various plant pathogens viz., fungi, virus, bacteria, phytoplasma etc, which are regularly used by researchers to reveal various facts regarding their pathogenecity and virulence. Besides these, there are databases solely devoted to plant pathology research.

Comprehensive Phytopathogen Genomics Resource (CPGR): This database was created to cater the requirement of 806 genome and trancriptome sequences of plant pathogens *viz*., bacteria, fungi, oomycete, nematode, virus and viroid, to plant pathologist and other researchers in the field of plant pathology (13). Evolution of diagnostic molecular markers and hefty diagnostic protocols for plant pathogens through employment of genomics is the major objective of this database.

Plant Resistance Genes Database (PRGdb): It is a web accessible open-source database which holds information about not less than 16 000 R-genes (resistance genes) in 192 plant species defied by 115 pathogens belonging to diverse groups (14). PRGdb is the first database of its kind dedicated solely for R-genes in plants, which are one of the most important genes in resistance breeding that can significantly reduce losses caused by pathogens.

PathoPlant: A new database, PathoPlant, combining information of specific plant-pathogen interactions on organism level and data about signal transduction on molecular level related with plant pathogenesis has been developed. It is a web-based service and the entries in the database are chained to associated in-house records as well as to entries in extrinsic databases such as SWISS-PROT, GenBank, PubMed, and TRANSFAC. At the present time, Patho Plant stores information regarding 104 plant-pathogen interactions in relation to 47 plant species and 29 pathogens, with the corresponding references and hyperlinks annotated (15).

Application of Bioinformatics in plant pathology research : With the help of genomic information of plant pathogens, stored in the databases, various tasks can be carried out through bioinformatics tools which are nothing but computer programs or algorithms. A vast array of computer programs are available, many of which are freely accessible in the web.

Comparative genomics to ascertain variability in the isolates of phytopathogens : In plant pathology, determination of genetic variability among isolates of plant pathogen is a routine work but the morphological variation gives only an idea of the genetic variability. Sequencing of plant pathogens followed by comparison with sequences of other isolates, utilizing bioinformatics tools, gives reliable and clear cut evidence of genetic variability. An algorithm, Basic Local Alignment Search Tool (BLAST) is one such program commonly used by Plant Pathologist for rapidly aligning and correlating a DNA or protein sequence in question with a database of sequences (16). The contrast of

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nucleotide or protein sequences to the sequence databases and the significance of matches are evaluated statistically, which thereby determines the underlying functional and evolutionary relationships existing in the sequences and also helps to identification of the members of gene families (17). Genome sequencing and comparative genomics have been utilized in study of various plant pathogens like *Rhizoctonia solani* (18), *Puccinia graminis* f.sp. *tritici* (19), etc.

Prediction of genes in the genomic sequence of phytopathogens : Analysis of the genes constituting the biological sequence of the organism *i.e.* gene prediction can be executed through bioinformatics using algorithms like BLAST, GeneScan, GeneMark, Glimmer, GeneBuilder, Grail etc (20) based on previously available gene sequence database of various phyotpathogens which have been experimentally verified. Many genes are discovered with additional help by comparison with known genes from other organisms, or searching for a match to a known protein sequence and the most popular tool to align genetic or protein sequences is BLAST. Zhou *et al.* (21) identified 306 new CDSs (Coding DNA sequence) in phytopathogenic bacteria *Xanthomonas campestris* involving a connected policy of bioinformatic, postgenomic and genetic approaches.

Bioinformatics approach in designing primers for PCR based diagnosis of plant pathogens : Nucleic acid based/ molecular method i.e. Polymerase Chain Reaction (PCR) method is one of the most reliable, accurate methods of plant pathogen detection and diagnosis utilizing primers, specifically fashioned for detection of plant pathogens. These specific primers for plant pathogens are designed based on genomic information of the pathogen in databases. Some of the online primer design sites are Gene Fisher, Do Primer, Primer 3, Web Primer etc (22). For accelerated and meticulous detection of three *Xanthomonas* species *viz*., *X. axonopodis* pv. *poinsettiicola* (XAP), *X. hyacinthi* (XH) and *X. campestris* pv. *zantedeschiae* (XCZ), Back *et al.* (23) developed a species-specific PCR assay and the species-specific primers were created from variable regions of the draft genome sequence data.

Phylogenetic analysis of phytopathogens through Bioinformatics approach : The evolutionary history based taxonomical classification of organisms, *i.e.,* Phylogenetics, an elemental part of the science of systematics that utilizes characteristics of organisms to create their phylogeny. Using bioinformatics approach, information on evolutionary relationships could be obtained through DNA sequence analysis, using those genes which are universally present and play essential role as these genes are likely to be highly conserved allowing little change over large span of evolutionary time, which are 18S rDNA in eukaryotes and 16 S rDNA in prokaryotes. Comparative analyses of 582 genomes across 75 genus groups, obtained from the NCBI genomes database in a high throughput approach was carried out utilizing the software EDGAR (Efficient Database framework for comparative Genome Analyses using BLAST score Ratios) and the results were integrated into the database. A quick survey of evolutionary relationships is executed through the software, simplifying the process of attaining new biological insights into the differential gene content of corresponding genomes (24).

Bioinformatics in fungal taxonomy in species identification : In fungal taxonomy, various techniques are being implemented for fungal identification and resolving the taxonomic positions. Of the various methods, Ribosomal DNA (rDNA) sequences is one of the most useful techniques aiding in fungal taxonomy. The three regions coding for the 5.8S, 18S, and 28 S rRNA form the ribosomal gene cluster. 5.8 rRNA gene, parts of the Large Subunit rRNA (LSU) 1 and 2 regions and Small Subinit rRNA (SSU) genes are useful for delineating of higher taxonomic ranks, such as classes and phyla and others for separation of genera and species (e.g. Internally Transcribed Spacer (ITS) 1 and 2 regions). In fungi, identification of species and molecular phylogeny, sequence analogy of the ITS region is extensively utilized (25) due to high copy number of rDNA

genes, which makes its amplification easier even from minute quantities of DNA and also, high degree of variation in present between closely related species. “FHiTINGS”, an open source software program, for rapid identification, classification and analyzing of ITS DNA sequences produced in fungal ecology studies utilizing the output of a BLASTn (blastall) search through next-generation DNA sequencing (26). Public databases like GenBank, EMBL, DDBJ and Molecular Mycology Research Laboratory provide a huge amount of ITS sequences of fungi for species and race identification.

Bioinformatics in group and sub group identification of Phytoplasma : Phytoplasma, cell wall less bacteria, inhabiting in phloem of infected plants, are responsible for causing numerous plant diseases. Being obligate in nature and unculturable *in vitro*, the launch of web based research tool, *i*PhyClassifier provided greater scopes for rapid recognition and classification of varied population of phytoplasma into groups and sub groups on the basis of restriction fragment length polymorphism (RFLP) pattern similarity coefficient scores (21).

Bioinformatics in the study of plant pathogen interaction : In cellular functions like initiation of pathogenesis and maintaining infection, a major role is being played by protein-protein interactions (PPIs). An understanding of the molecular basis of pathogenesis is primarily based to a certain extent on the PPI network between a host and pathogen. Such informations can be best utilized for management of diseases through designing of pesticides which could target specific pathogen protein so as to avert disease inducing harmful interaction. For the purpose of experimental characterization of the molecular dynamics of plant-pathogen interactions, a model system was adopted where a major bacterial leaf pathogen, *Pseudomonas syringae* pv. *tomato* strain DC3000 (*PstDC3000*) was asserted to infect the plant host *Arabidopsis thaliana* (27). An internet-based database, PHI-base, classifies experimentally verified pathogenicity, virulence and effector genes from fungal, Oomycete and bacterial pathogens, which infect animal, plant, fungal and insect hosts. It has the potential in the exploration of genes in agronomically important pathogens, which might be the targets for chemical intervention (28).

Protein-protein docking : In an endeavor for identification and cataloging of physical interactions between pairs or groups of proteins, a combination of bioinformatics and structural biology is utilized through prediction of interaction between protein–protein. An understanding of protein–protein interactions (PPIs) is of prime importance in the attempt for scrutiny of intracellular signaling pathways and modelling of protein complex structures. There is a close relation between the fields of protein–protein interaction prediction and protein–protein docking, which attempts to employ geometric and steric considerations to fit two proteins of recognized structure into a bound complex. Prognosis of the three dimensional structure of the macromolecular complex of interest which would occur in a living organism is the eventual objective of docking. The most widely used Protein-Ligand docking softwares are AutoDock, AADS, Surflex, VoteDock, SODOCK, SwissDock etc (29).

Docking as a tool against plant viruses : Docking, a method utilized for determination of the desired orientation of one molecule to a second when both are attached to each other forming a stable complex (30), which could be utilized for studying the mechanism of an enzymatic reaction, recognition of all possible attachment modes for ligands, screening a database and prognosis of ligation position of small molecule or anti viral agents to their object proteins (31). Docking acts a powerful resource for structure based drug discovery (32). A study on homology modeling and docking in *Mimosa yellow vein virus* resulted in screening of inhibitors against the virus protein and opened up scopes for designing antiviral agents against *Geminiviruses* in the future (33).

Secretome analysis in fungal pathogens : Secretome, the secreted proteins, is helpful in studying the underlying mechanisms involved in host pathogen interaction in case of fungal

Table 1. List of some important plant pathogens whose complete genome have been sequenced

Scientific Name	Disease caused	Genome Size	Reference
Plant pathogenic Fungi			
Magnaporthe grisea	Rice blast	40 Mb	Dean *et al*. (37)
Ustilago maydis	Smut	20.5 Mb	Kamper *et al.*(38)
Fusarium oxysporum f. sp. *lycopersici*	Wilt	60 Mb	Ma *et al*. (39)
Botrytis cinerea	Grey mold	38.8 Mb	Amselem *et al*. (40)
Sclerotinia sclerotiorum	White mold	39.6 Mb	Amselem *et al*. (40)
Puccinia graminis f. sp. *tritici*	Black rust	89 Mb	Duplessis *et al*. (41)
Mycosphaerella graminicola	Blotch	33.6 Mb	Goodwin *et al.*(42)
Colletotrichum graminicola	Anthracnose	57.4 Mb	O'Connell *et al*. (43)
Macrophomina phaseolina	Charcoal rot	49 Mb	Islam *et al*. (44)
Fusarium graminearum	Head scab	36 Mb	King *et al*. (45)
Plant Pathogenic Bacteria			
Xylella fastidiosa	Pierce's disease, Citrus variegated chlorosis disease	2,679,306 bp	Simpson *et al.*(46)
Agrobacterium tumefaciens	Crown gall	2,841,581 bp	Goodner *et al*.(47)
Ralstonia solanacearum	Bacterial wilt	3,716,413 bp	Salanoubat *et al*. (48)
Xanthomonas axonopodis pv. *citri*	Citrus canker	5,175,554 bp	da Silva *et al*. (49)
Xanthomonas campestris pv. *campestris*Black rot of crucifers	5,148,708 bp	Qian *et al*. (50)	
Pseudomonas syringae	Blight, wilt	6,093,698 bp	Feil *et al*.(51)
Xanthormonas oryzae pv. *oryzae*	Bacterial blight of rice	4,941,439 bp	Lee *et al*. (52)

pathogens. Genome sequencing and various bioinformatic analysis are carried out to predict secretomes produced by the fungi in establishing infection in their host. But all-inclusive secretomic profiles of fungal pathogens are lacking as the process is in the budding stage. Predicted secretomes have been used in interspecies comparative analysis of important fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea* causing necrosis in plants (34). Analysis of the secretomes in plant pathogenic fungi *Fusarium graminearum* (35), *Phytophthora plurivora* (36) etc have been carried out for understanding the association of the pathogen with their host plants.

Conclusion

Bioinformatics has a tremendous potential to assist Plant Pathologists in their efforts to reduce crop losses due to diseases. Plant Pathologist around the world has already acknowledged this fact and bioinformatics has evolved to be an elemental part of plant pathology research. Bioinformatics tools give valuable insights into the mechanism of pathogenesis and also help in figuring out an explanation regarding the methods and mechanisms associated in

Table 2. List of some important bioinformatics software used in the study of plant pathogens

Tool name	Description	Web Address	Reference
BLAST (Basic local alignment search tool)	Program utilized for identifying regions of local similarity between nucleotide or protein sequences and comparing them to sequence databases and thereby calculating the statistical significance of matches	https://blast.ncbi.nlm.nih.gov/	Altschul *et al*. (16)
GeneFisher	Web-based program for designing degenerate primers in an user interaction mode	bibiserv.techfak.uni-bielefeld.de/genefisher2/	Giegerich *et al*. (53)
GENSCAN	A software to acquire a DNA sequence and find the open reading frames that accord to genes	genes.mit.edu/GENSCAN.html	Burge and Karlin (54)
GeneMarkS	A program for prokaryotic gene annotation	http://exon.gatech.edu/Genemark/genemarks.cgi	Besemer *et al*. (55)
ProbCons	A protein multiple sequence alignment program	https://toolkit.tuebingen.mpg.de/probcons	Do *et al*. (56)
MEGA 4 (Molecular Evolutionary Genetics Analysis)	A tool for operating automatic and manual sequence alignment in an integrated manner, inferring phylogenetic trees, mining web-based databases, evaluating rates of molecular evolution and testing evolutionary hypotheses	www.megasoftware.net/	Kumar *et al*. (57)
AutoDock	Docking tool, working in an automated manner devised for prediction of how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure	autodock.scripps.edu/	Trott and Olson (58)
RDP3	Program suitable for characterization of recombination events in DNA-sequence alignments	web.cbio.uct.ac.za/~darren/rdp.html	Martin *et al*. (59)
SwissDock	Web based service for prediction of molecular interactions that might occur between a protein in question and a small molecule	www.swissdock.ch/	Grosdidier *et al*. (60)
Primer3	Tool for designing and analyzing primers for PCR and real time PCR experiments	simgene.com/Primer3	Untergasser *et al*. (61)
*i*PhyClassifier	Internet-based search tool for rapid recognition and classification of varied phytoplasmas	plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi	Zhao *et al*. (62)

triggering of plant immunity which can assist in improving plant disease resistance. Field of computational biology particularly homology modeling and docking have the potential to discover various unconventional chemicals against many plant pathogens including plant viruses where no effective direct methods of control is available at present in field conditions. The achievements made in Plant Pathology through bioinformatics or computational biology are plenty, but this is just a tip of the iceberg and many more breakthroughs are yet to be made in near future.

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SCIENTIFIC NEWS

New UGC Regulation on board to categorize Indian Universities: In a significant development that will have a major impact on University education in India, the University Grants Commission (UGC) has approved a new set of Regulations called the UGC (Categorization of Universities for Grant of Graded Autonomy) Regulations, 2017. Under these, the Universities will be classified into three categories based on their National Assessment and Accreditation Council (NAAC) score and ranking under National Institute Ranking Framework (NIRF): Category I, Category II and Category III. The sole objective of the new regulation is providing greater autonomy to performing universities. Hence, the regulations have proposed that Category I and Category II institutions will be given greater autonomy in term of both academic and financial decisions and modalities.

Kalam satellite, World's lightest satellite launched by Tirupati boy: It was reported that Mr.K. Yagna Sai's, a native of Tirupati, who was part of the six-member team from Chennai-based Space Kidz India that designed KALAMsat, which was launched by NASA at Wallops, Virginia, on June 22. Mr.Sai is a B.Tech final year student at Hindustan University, Chennai, and Mr.Sai was the 'Lead Technician' in the team that designed the satellite termed as world's lightest (weighing a mere 64 gm) and also the first 3D-printed.

Northern River Terrapin, a critically endangered species gets new home: A critically endangered resident of the Sunderbans is set to get a new home, beginning a slow journey to recovery from a disastrous decline in the wild. It is more threatened than the Bengal tiger, but far less known. Before winter this year, three fresh water ponds in the Sunderbans Tiger Reserve will house the rare Northern river terrapin (Batagur baska), whose presence in the wild in West Bengal and Odisha had declined to undetectable levels a decade ago. Batagur baska, the 60-cm-long turtle that is presumed extinct in several Southeast Asian countries, is classified as critically endangered by the International Union for Conservation of Nature in its Red List of threatened species. The tiger, by comparison, is endangered.

HEERA will supersede UGC and AICTE: The University Grants Commission (UGC) and the All India Council for Technical Education (AICTE) will soon become history. The Sri.Narendra Modi government has decided to replace the two education bodies with one regulator. The new body will most-likely be called HEERA - Higher Education Empowerment Regulation Agency. The government has been mulling over the thought for quite some time now. Many experts had insisted for the radical change in education but the decision could not be taken. The final call was taken eventually in March at a meeting on education chaired by the Prime Minister. The human resource development (HRD) ministry and the Niti Aayog are working on the new law.

Goa to host science and art fete in November: The event will be held at various venues across the city from November 10 to19. Multiple projects will explore 'space' and its connection to science, philosophy, art and culture. Following the success of its first science-meets-art festival, The Story of Light, in Goa in 2015, its organisers are back this November with 'The Story of Space' festival. The Story of Space 2017 is an interdisciplinary, informal learning festival that will explore 'space' and its connection to science, philosophy, art and culture.

Novel Ways to Curb Metastasis: It was reported that when cancer cells get densely packed they secrete two proteins that deliver a stark message

to other cells: go away. This causes the cancer cells to break off from the pack and float through the blood stream or lymphatic system to other sites and start growing afresh. In the ongoing war against cancer, an international team led by scientists from Johns Hopkins University has found what causes the spread of cancer and what could slow it down (Nature Communications). This is important because 90% of cancer deaths are caused when cancer cells break off from the origin and start spreading elsewhere in the body. There are no existing drugs for stopping this spread, known as metastasis, of cancer. It was found that it was not the overall size of a primary tumor that caused cancer cells to spread, but how tightly those cells are jammed together when they break away from the tumor. And a medication mix that kept this microscopic message from being delivered and the two existing drugs, Tocilizumab and Reparaxin, prevented cancer cells from getting their marching orders. They discovered a new signaling pathway that, when blocked, could potentially curb cancer's ability to metastasize.

Nanoparticles from Curcumin provides new relief to TB Patients: Curcumin, the basic ingredient of turmeric, when administered in a nanoparticle formulation has several favourable properties in the treatment of tuberculosis in mice, and it was observed that nanoparticle curcumin to be five times more bioavailable (which is the proportion of drug that enters circulation after introduction into the body) in mice, than regular curcumin, and was able to drastically reduce liver toxicity induced by TB drug isoniazid. More importantly, treatment of TB with isoniazid along with 200 nanometre curcumin nanoparticles led to "dramatically reduced" risk of disease reactivation and reinfection. Treatment with anti-tuberculosis drugs takes about six-nine months in the case of drug-sensitive TB and 12-24 months for drug-resistant TB. Curcumin blocks the Kv1.3 potassium channel and prevents apoptosis, or cell death, of T cells that come up with an immune response. As a result, the protective, long-lasting memory cells called the central memory T cells get enhanced. Mice treated with curcumin nanoparticles and isoniazid were able to clear the bacteria at an accelerated rate in both the lungs and spleen.

Novel vaccine that lowers Cholesterol: A cholesterol-lowering vaccine has shown promise in mice, said researchers who announced they had started early-phase trials to see if it also works in humans. Such a treatment could offer a welcome alternative to widely used statins, the main pharmaceutical choice today for lowering cholesterol in people at high risk of heart attack or stroke. Early-phase trials checking if antigen, positive in mice, works in humans. The vaccine, dubbed AT04A, reduced cholesterol levels in trial mice by half, and reversed damage done to blood vessels due to plaque build-up by more than 60%, researchers said in a statement. The mice were given the vaccine after they were fed a fatty diet to resemble the high-cholesterol intake of a human Western-style diet. Levels of cholesterol were reduced in a consistent and long-lasting way. This resulted in a reduction of fatty deposits in the arteries and atherosclerotic damage, and reduced arterial wall inflammation. Atherosclerosis occurs when a waxy compound lines blood vessel walls, limiting blood flow and potentially triggering dangerous blood clots.

Photosynthesis may cure Heart disease: Scientists have found that using blue-green algae and light to trigger photosynthesis inside the heart could help treat cardiac disease, the top cause of death globally. Researchers injected a type of bacteria into the hearts of anaesthetised rats with cardiac disease. Using light to trigger photosynthesis, they were able to increase the flow of oxygen and improve heart function. The beauty of it is that it's a recycling system. The genesis of this somewhat mind—boggling concept

sprang from scientists searching for new ways to deliver oxygen to the heart when blood flow is restricted. The next round of experiments involved injecting the cyanobacteria into the beating hearts of anaesthetised rats with cardiac ischemia. They then compared the heart function of rats with their hearts exposed to light (for less than 20 minutes) to those who were kept in the dark. The group that received the bacteria plus light had more oxygen and the heart worked better. The bacteria dissipated within 24 hours, but the improved cardiac function continued for at least four weeks, he said. The researchers plan to investigate how to apply this concept to humans and how to deliver a light source to the human heart. They are also examining the potential of using artificial chloroplasts to eliminate the need for bacteria.

Genetic basis for longer life traced to Sharks: Greenland sharks, the longest living vertebrates on Earth which live for up to 400 years, could hold the secret to long life, geneticists mapping their DNA say. The sharks are believed to have unique genes which could help explain not only their incredibly long life span, but life expectancy in other vertebrates, including humans. The researchers sequenced the DNA from Greenland sharks, some of which were alive in the Georgian era. They are now searching for the 'unique genes' which could hold the secret to the shark's longevity. They sequenced the full mitochondrial genome (the complete mitochondrial DNA information of an organism) of almost 100 Greenland sharks, which includes individuals born in the 1750s. Since the Greenland shark lives for hundreds of years, they also have enough time to migrate over long distances and our genetic results showed exactly that. Most of the individuals in our study were genetically similar to individuals caught 1000s of kilometres away.

Genetics justifying the Aryan Migration doctrine: New DNA evidence is solving the most fought-over question in Indian history. Genetic research based on an avalanche of new DNA evidence is making scientists around the world converge on an unambiguous answer. It was stated that Genetic influx from Central Asia in the Bronze Age was strongly male-driven, consistent with the patriarchal, patrilocal and patrilineal social structure attributed to the inferred pastoralist early Indo-European society. This was part of a much wider process of Indo-European expansion, with an ultimate source in the Pontic-Caspian region, which carried closely related Y-chromosome lineages. across a vast swathe of Eurasia between 5,000 and 3,500 years ago. The scientists state that the prevalence of R1a in India was very powerful evidence for a substantial Bronze Age migration from central Asia that most likely brought Indo-European speakers to India.

Novel drug delivery system to kill cancer cells: Researchers at Indian Institute of Science Education and Research, Pune have successfully developed a novel cancer drug delivery system using graphene oxide nanoparticles. In a serendipitous discovery, they found that when a FDA-approved anticancer drug cisplatin was added, the graphene oxide sheets self-assembled into spherical nanoparticles enclosing the drug within. We were very surprised to see this kind of shape-shifting transformation of the graphene oxide sheets into a spherical structure. We are exploring the mechanism by which this happens. They stated that the drug is reacting with graphene oxide and transforming the graphene sheet into a ball-like structure, a kind of 'molecular stitching. The nanoparticles of 90-120 nanometre size containing cisplatin and either of the two anticancer drugs were taken up by cervical cancer cells leading to programmed cell death. The nanoparticle containing cisplatin alone was able to kill cancer cells. But there is additive effect when two drugs are used together and efficiency of killing the cancer cells becomes better. The drugs bind to the DNA strands and break the strands so cell division does not happen and programmed cell death ensues.

Superalgae to combat Coral bleaching: Researchers have found a solution to reduce coral bleaching by genetically engineering the micro-algae found in corals, enhancing their stress tolerance to ocean warming. These micro-algae are called Symbiodinium, a genus of primary producers found in corals that are essential for reef health and, thereby, critical to ocean productivity. Different species of Symbiodinium have large genetic variation and diverse thermal tolerances which effect the bleaching tolerance of corals. The researchers used sequencing data from Symbiodinium to design genetic engineering strategies for enhancing stress tolerance of Symbiodinium, which may reduce coral bleaching due to rising ocean temperatures. Very little is known about Symbiodinium, thus very little information is available to improve coral reef conservation efforts.

Earth turning out into a Plastic Hub: Humans have created 8.3 billion metric tonnes of plastics since early 1950s, and most of it now resides in landfills or the natural environment, a study has found. Researchers found that by 2015, humans had generated 8.3 billion metric tonnes of plastics, 6.3 billion tonnes of which had already become waste. Of that total waste, only 9 per cent was recycled, 12 per cent was incinerated and 79 per cent accumulated in landfills or the natural environment, researchers said. If current trends continue, roughly 12 billion metric tonnes of plastic waste will be in landfills or the natural environment by 2050, researchers said.

Anti Malarial drug enshields foetus from Zika infection: Commonly used malaria drug hydroxychloroquine can effectively block the Zika virus from crossing the placenta and getting into the foetus and damaging its brain, as reported by the researchers from Washington University School of Medicine. The drug already has approval for use in pregnant women. The placenta acts as a barrier to protect the developing foetus from disease-causing organisms. It prevents pathogens from reaching the foetus through a form of a garbage recycling system that removes some components of cells, termed autophagy. It was observed that the Zika virus actually manipulates the garbage recycling system to its own advantage. The Zika infection ramps up autophagy. So when we use a drug that inhibits or suppresses this ramping up, we can block the virus from infecting the foetus. (The Journal of Experimental Medicine). Pregnant mice infected with Zika virus was treated with the drug or a dummy for five consecutive days. Compared with the controls, there was significantly less virus in the placenta of mice that received the drug.

Massive seed production of Indian Pompano: The regional centre of ICAR-Central Marine Fisheries Research Institute has made a major breakthrough by undertaking mass scale seed production of Indian pompano for the first time in the world. Indian pompano (*Trachinotus mookalee*) is a marine fish belonging to the family Carangidae. It is low in landing from the wild. It contains Omega 3 and 6 fatty acids.

Obituary : Renowned Space Scientist Prof. UR Rao, Passed away on 24th July, 2017. The former ISRO Chief was serving as the Chairman of the Governing council of the physical research laboratory and the Chancellor of the Indian Institute of Science and Technology at Thiruvananthapuram. From Aryabhatta to the Mars Orbiter Mission, Prof.Rao has continued to work with the space agency on several of its projects.

Prolific Physicist and Educationist Prof.Yash Pal, is a Scientist, communicator and institution builder, passed away on 25th July, 2017. Belonging to an era of scientists who espoused 'Make In India' decades before it became a politico-marketing cliché. Prof.Pal has began his career as a Professor at the TIFR. A scientist of international repute, Prof.Pal was awarded the

Padma Bhushan in 1976 and India's second highest civilian honour, the Padma Vibhushan, in 2013. He made significant contributions in the field of science and to the study of cosmic rays, high-energy physics, astrophysics. He served as UGC chairman and leads a committee for Higher Education reforms in India and advocated to establish a single regulator.

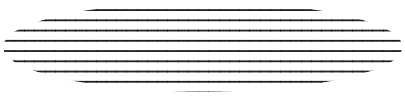
OPPORTUNITIES

International centre for Genetic Engineering and Biotechnology-Post Doc Fellowships: ICGEB offers competitive Postdoctoral Fellowships in Life Sciences to highly motivated scientists wishing to pursue postdoctoral research in a world-class scientific environment. Refer web site: https://www.icgeb.org/postdoc-application.html. Closing Dates for Applications 31 March and 30 September (two times a year).

IISER Pune Postdoctoral Research Associate: Applications are invited for Postdoctoral Research Associate (PRAs) positions at the Indian Institute of Science Education and Research (IISER) Pune, India. These positions are open for candidates with 0-5 years of experience after the submission of their PhD thesis. Candidates may apply by email by sending the following documents to postdocapplications@iiserpune.ac.in and refer web site http://www. iiserpune.ac. in/links / postdoctoral-research. Complete applications would be considered in March and September of each year; the deadlines are March 1 and September 1 (two times a year).

SERB-National Post Doctoral Fellowship (N-PDF): The SERB-National Post Doctoral Fellowship (N-PDF) is aimed to identify motivated young researchers and provide them support for doing research in frontier areas of science and engineering. The fellows will work under a mentor, and it is hoped that this training will provide them a platform to develop as an independent researcher. The call for applications for SERB-N PDF will be notified twice a year (September and March) through the website www.serbonline.in and www.serb.gov.in.

The Directorate for Biological Sciences (BIO): The Directorate for Biological Sciences (BIO) awards Postdoctoral Research Fellowships in Biology to recent recipients of the doctoral degree for research and training in selected areas supported by BIO and with special goals for human resource development in biology. Contact, Michael J. Vanni, email-bio-dbi-prfb@nsf.gov and Diane Jofuku Okamuro, email- dokamuro@nsf.gov. Full Proposal Deadline Date, November 7, 2017.



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