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(Regn. No. 28 OF 2007)

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Optimization of Polyhydroxybutyrate (PHB) Production by Locally Isolated *Bacillus aryabhattai* Using Response Surface Methodology

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Abstract

In the present study, an attempt was made to isolate an efficient Polyhydroxybutyrate producing bacterium from soil. A total of 38 different types of bacteria were isolated, out of which 15 were found to be PHB positive, based on the viable colony staining method of screening using Nile red Dye. The isolate (6N) showed maximum PHB production of 0.17 g/L, and PHB produced was confirmedusing NMR.The most potent isolate (6N-NRC) was identified using 16S rRNA, and phylogenetic analysis clearly demonstrated that the strain 6N-NRC is a member of the genus *Bacillus* and is identified as *Bacillus aryabhattai*. The culture medium and growth parameters were optimized using one factor at a time, multifactorial experimental design (Plackett-Burman and Box-Behnken) and utilization of Beet molasses as cheap and economic carbon source for maximum PHB production was done. Beet molasses (30 g/L) as the carbon and ammonium chloride (0.75 g/L) as the nitrogen source were found to be the best nutritional sources for maximum PHB production. Incubation time period 36h, pH of the medium at 8.0 and temperature of 30^0C were found to be optimum conditions for obtaining maximum PHB yield of 3.799 g/L.

Key words : Polyhydroxybutyrate (PHB), NMR, *Bacillus aryabhattai*, multifactorial experimental design, Beet molasses.

Introduction

Plastics like polypropylene, polyethylene and polystyrene are almost made from petroleum which is the main source of energy (1). Around 270 million metric tons per year of fossil fuels are consumed for manufacturing plastics (2). The consumption of petroleum at this rate will lead to its depletion in the next 60-80 years (3). On the other hand, the environmental accumulation of plastics has become a worldwide problem (4).

Consequently, petroleum based plastics were replaced by biodegradable polymers which are considered ecologically as useful alternatives to plastics (5). Poly-3-hydroxy butyric acid (PHB)is the common type of polyhydroxy alkonoates (PHA) from which bioplastics are made (6). PHB has unique properties such as UV resistant, insoluble in water, oxygen permeability and highly resistant to hydrolytic degradation, poorly resistant to acids and bases, soluble in chloroform and other chlorinated hydrocarbons and thus it is used in medical applications (7). PHBs are considered as to be sources for

biodegradable and biocompatible plastic materials (8). Thus, the current problems causedby decreasing the nonrenewable energy resources and environmental pollution caused by plastic garbage are reduced. PHB are also used in material science, food industries and agriculture (9).

A large number of microorganisms have the ability to synthesize PHB as intracellular energy reserve material under certain limitations of some essential nutrients such as nitrogen, magnesium and phosphate in presence of excess carbon sources (10). Many kinds of microbes such as *Bacillus* sp. (11), *Pseudomonas* (12), *Azotobacter- vinelandii* (13), *Sinorhizobium meliloti* and *Escherichia coli* (13, 14) are able to generate PHB.

The production of PHB either on the industrial or commercial scale is limited due to the relatively high cost of the utilized substrate compared to synthetic plastic (9).Hence, the strategy of biodegradable plastic production is important for PHB production as it relies on isolating most efficient PHB producing bacterial strain and optimizing the cultural parameters (5).

The purpose of this work was to isolate and identify a local bacterial strain capable of producing PHB followed by identification of the best isolate by 16s rRNA sequence analysis and phenotypic characterization. The one-factor-at-a time experiments were done to choose the optimum carbon and nitrogen sources. Then, Plackett-Burman design was used to identify the most significant variables affecting production of PHB. Moreover, Box-Behnken was done to optimize the effective variables. Furthermore, the chemical structure of PHB synthesized by the most potent isolate was determined by NMR spectroscopy analysis.

Materials and Methods

Sample collection and isolation of pure cultures: Microbial isolates were recovered from six different soil samples originating from different Egyptian cities and given the symbols as follows: El Menoufia (A), El Wadi El Gedid (E), El Fayoum (G)and El Giza (J, K and N). One gram of soil sample is dispensedin 10mls of sterile distilled water, mixed vigorously and 1ml from this is taken and added to another tube with 9mls sterile distilled water to get a dilution of 10^{-1}. This serial dilution is repeated to get dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, $10^{-6}$and 10^{-7}. For the isolation of organisms, 0.1ml of each dilution was plated onto a nutrient agar medium by spread plate method for the propagation of microbial growth. The plates were incubated at 30°C for 48 hours. Colonies with different characteristic features were maintained as pure cultures on nutrient agar slants and stored at 4°C (15).

Screening of PHB producing isolates by nile red dye: All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Nile red Dye. For this screening of PHB producers, 20 µl was spread onto sterilized pre-made (minimal salt agar media) plates to reach a final concentrations of 0.5 µg Nile red /ml medium. After inoculation, the plates were incubated overnight at 30 ºC subsequently. The prepared clay soil samples were subcultured by 0.1 ml samples and then spread out with a sterilized glass rod over the surface of minimal salt agar media. The plates were incubated at 30 ºC for 48h. Colonies with pinkish pigment indicated PHB production isolates were exposed to ultraviolet light (312 nm) to detect the accumulation of PHB according to the lighted plates and were given positive signs. After that these isolates were picked up and purified by sub-cultured on the same media (16).

Determination of cell dry weight: After 48h incubation at 30°C, culture medium was collected and the cell dry weight was measured by centrifugationof 100ml of the culture at 10,000 rpm for 15min at 4ºC. Supernatant was discarded and the cell pellets werewashed twice in deionized water and dried at 80ºC until a constant weight then the total bacterial cell dry weight was determined as g/l (17).

Production medium: Minimal Salt Media (MSM) (18) was prepared as follows:(KH_2PO_4 1.5g; Na_2HPO_4 3.525g; $MgSO_4.7H_2O$ 0.2g; $CaCl_2$0.02g; ferric citrate 0.0015g; glucose 20g; NH_4Cl 0.75g; trace elements solution 1ml; distilled water 1000 ml; pH 7.5) for the production of poly- β–hydroxybutyrate. MSM (100 ml) was taken in each Erlenmeyer flask and autoclaved at 121°C for 20 min after which 10% (v/v) of fresh bacterial inoculum were inoculated in each flask and incubated for 7 days at 30°C.

Extraction and quantification of PHB: Polyhydroxybutyrate polymer was extracted and the amount of PHB produced was calculated from the standard curve prepared by using commercial poly-β-hydroxybutyrate (Sigma-Aldrich) as per the method detailed by Law and Slepecky (19). All the PHB positive bacterial isolates were inoculated in minimal salt medium and the cell growth of each isolate containing the polymer was centrifuged at 10,000 rpm at 4°C for 10 min. The pellet was washed with acetone and ethanol to remove the unwanted materials, resuspended in equal volume of 4 % sodium hypochlorite and incubated at 37°C for 24h. The mixture was then centrifuged at 10,000 rpm for 10 min to sediment the lipid granules. The supernatant was discarded, and the cell pellet was washed successively with acetone, ethanol and water to remove unwanted materials. The whole mixture was centrifuged again and the supernatant was discarded. Finally, the pelleted polymer granules were dissolved in hot chloroform and filtered through Whatmann no. 1 filter paper (previously treated with hot chloroform). To the filtrate, 10 ml of hot concentrated H_2SO_4 were added, which converts the polymer to crotonic acid, turning it into a brown colored solution. The solution was cooled and absorbance was read at 235nm against a concentrated H_2SO_4 blank on UV-VIS spectrophotometer. The quantity of PHB produced was determined by referring to the standard curve (5).

Molecular identification of selected PHB producing bacterial isolate: The genomic deoxyribonucleic acid (RNA) was extracted from isolated culture of *Bacillus* strain (6N-NRC) by using the protocol of Gene JET Genomic DNA Purification Kit (Thermo K0721,Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA). The PCR amplification of 16S ribosomal gene was performed by using Maxima Hot Start PCR Master Mix (Thermo K1051) and the nucleotide sequences of the 16S primersused are: forward primer-5'-AGAGTTTGATCCTGGCTCAG-3'and reverse primer-5' GGTTACCTTGTTACGACTT -3' (20). To each PCR vial containing 10 µL of 2X PCR Master Mix, 2 µL of each used primer (10 pmole/µl) and 2 µL of the purified DNA sample (40 ng/ µl) were added. The total volume of the amplification reaction was completed to 20 µl using sterile distilled water. The amplification protocol was carried out as follows: Denaturation at 95°C for five min. Thirty-five cycles each consists of the following segments: Denaturation at 95°C for one min; primer annealing for two min. at 52°C and polymerization at 72°C for two min. Finally, hold the PCR at 4°C. The PCR product was cleaned up using Gene JET™ PCR Purification Kit (Thermo K0701). The DNA sequencing of the PCR product was carried out by using Applied Biosystems (ABI) 3730xl DNA sequencer (GATC Biotech, Constance Germany) by using forward and reverse primers.

Phylogenetic analysis : The 16S ribosomal DNA (rRNA) sequences of the strain (6N-NRC) compared with the sequences available in National Center for Biotechnology Information (NCBI), Gene Bank database by using the Basic Local Alignment Search Tool (BLAST). The sequences were aligned together with those of reference taxa retrieved from public databases. The evolutionary distances were set up based on parameter model and phylogenetic tree was constructed by using the neighbor-joining method MEGA6 software (21, 22). The 16S rRNA sequence was submitted to the NCBI Gene Bankwith nucleotide sequence database under accession number MH997667.1

Nuclear magnetic resonance (NMR) analysis: The proton Nuclear Magnetic Resonance

Spectroscopy (^{1}H NMR) of the polymer was recorded after suspending the polymer in high purity deuterochloroform ($CDCl_3$). 1H NMR spectra were obtained in model Bruker High Performance Digital FT-NMR spectrometer AvanceIII 400 MHz at 20-25°C, 4.0894465s acquisition time and 8012.820 Hz spectral width.

Optimization of cultural parameters for maximum PHB production: Different factors affecting PHB production by the selected bacterial isolate were optimized using one factor at a time and multi-factorial design techniques.

Optimization using one factor at a time (OFAT)

Effect of different incubation periods: The bacterial isolate was grown in flask (250 ml) with 100 ml minimal salt medium at pH 7.0 and was sterilized at 121°C for 20 min The inoculated flasks with 10% v/v were incubated at 30°C at 200 rpm under different incubation periods (1,2,3,4, 5,6,7, 8,9 and 10 days), PHB produced was quantified.

Effect of different carbon sources : The effect of different carbon sources on PHB production was determined by inoculating the bacterial isolate in 100 ml of minimal salt medium (MSM) (18) supplemented with different carbon sources such as glucose, fructose, sucrose, maltose, arabinose, galactose and gluconic acid at 2% concentration. Cheap carbon sources like sugarcane and beet molasses were also tested as carbon sources. Cultures were incubated at 30°C on a rotary shaker (200 rpm) for 48 h. After incubation, PHBs produced were quantified.

Effect of different nitrogen sources: The bacterial isolate was grown in 100 ml of MSM broth containing the optimum carbon source and different organic and inorganic nitrogen sources (ammonium sulphate, ammonium chloride, ammonium nitrate, urea, casein, yeast, corn steep liquor, beef extract and peptone) were used at nitrogen base concentration. After 48 h of incubation at 30°C, PHB yield was determined, and the optimum nitrogen source was selected.

Effect of different inoculum size: Different inoculum sizes (1,2,3,4,5,6,7,8,9 and 15 %v/v) of bacterial isolate were grown in 100 ml of MSM broth. After 48 h of incubation at 30°C, PHB yield was determined, and the optimum inoculum size was selected.

Statistics: All experiments were performed in triplicates, the data shown in the corresponding tables and figures were the mean values of the experiments and the relative standard deviations were shown (mean ± SE).

Multifactorial experimental design and optimization: Experimental design as two steps sequential optimization is used to screen many variables together in one experiment and to optimize them for a desired response in a much faster way than examining one variable at a time.

Plackett-Burman design : Plackett-Burman Design (PBD) was employed for selection of significant variables in PHB production. Application of statistical methods involving Plackett-Burman Design (PBD) has gained a lot of impetus for medium optimization (23). Plackett-Burman design was used to screen the most significant parameters affecting PHB production. This design is recommend when more than five factors are under investigation (24). Seven independent variables were screened in nine combinations, organized according to the Plackett-Burman design matrix. For each variable, a high level (+) and low level (-) was tested. All trials were performed in triplets and their averages were treated as the responses.The main effect of each variable was determined by the following equation:

$$E_{xi} = (\ _{Mi+} - \ _{Mi-}) / N$$

Where E_{xi} is the variable main effect, Mi+ and Mi- are either PHB production in g/l or dry cell weight of the selected isolate or PHB yield % in trials where the independent variable (xi) was present in high and in low settings, respectively, and N is the number of trials divided by 2.

Box-Behnken design: In the second phase of medium formulation for maximum PHB production,

the Box-Behnken experimental design was applied where the most significant independent variables, named (X_1), (X_2) and (X_3) were included and each factor was examined at three different levels, low (-), high (+) and central or basal (0). Thirteen combinations and their observations were fitted to the following second order polynomial mode:

$$Y = b_o + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

Where, Y is the dependent variable (PHB production); X_1, X_2 and X_3 are the independent variables; b_o is the regression coefficient at centerpoint; b_1, $b_2$and b_3 are linear coefficients; b_{12}, b_{13} and b_{23} are second-order interaction coefficients; and b_{11}, b_{22} and b_{33} are quadratic coefficients.The values of the coefficients were calculated and the optimum concentrations were predicted using JMP software. The quality of the fit of the polynomial model equation was expressed by R^2 (regression coefficient). If the proposed model is adequate, as revealed by the diagnostic tests provided by an analysis of variance (ANOVA) and residual plots, contour plots can be usefully employed to study the response surface and locate the optimum operational conditions (25, 26). The 3D graphs were generated to understand the effect of selected variables individually and incombination to determine their optimum level for maximal PHB production. The F-test was performed to determine factors having a significant effect ($P < 0.1$).

Results and Discussion

Isolation and screening of PHB producing microorganisms: In this study microorganisms were isolated from clay soilsamples using serial dilution. Atotal of 38 bacterial colonies with different morphological features were selectedand given numbers.Bacterial colonies were preserved on enriched nutrient agar medium to be studied (Table 1). Among 38 colonies, 15 colonies showed positive pinkish colony for Nile red staining. The bacterial strains were further evaluated for PHB production by preliminary screening using submerged fermentation technique; quantification was done spectrophotometrically and by comparing the absorbance readings with a standard crotonic acid curve. The cell dry weight (g/L) and PHB yield % were studied for the 15 positive isolates (Table 2). The colonies of the most potent isolate (6N) grown on minimal salt medium containing Nile red dye under ultraviolet light (UV) showed pink fluorescence which indicated the presence of PHB. The PHB yield % ranged from 12.8-38.63% and the highest percentage was observed by the isolate designated as 6N. Bacteria belonging to *Bacillus* were known previously to accumulate high concentrations of PHA (27, 28). Similar results were obtained by Aly*et al.* (6) who studied the production of PHB using *B. cereus* MM7 isolated from soil samples. Higher results were shown by Bhuwal *et al.* (29) who estimated the maximum PHA production was 79.27% and 77.63% using *Enterococcus* sp. and *Brevundimonas* sp. respectively isolated from cardboard industry waste water. In literature, both gram positive and gram negative bacteria are capable to accumulate PHB (30). Similar results show that the natural environment has been an extensive area of research for the production of PHB (31). Different environments including soil (32), sea water and deep sea mud (33)and oil sludge (34) represent rich areas for extensive screening and identification of gram positive and gram negative bacteria for PHB production.

Molecular characterization of the selected isolate: 16S rRNA gene investigation was performed. The 16S forward and reverse primers were used to amplify the region of the 16S ribosomal ribonucleic acid (rDNA) gene from the genomic DNA of the *Bacillus* (6N-NRC). After the amplification by PCR, a product of nearly 1500 bp was obtained. The BLAST analysis of the amplified 16S rDNA gene sequence revealed 99% similarity to the partial 16S rDNA gene of *Bacillus aryabhattai* B8W22 strain. Phylogenetic analysis clearly demonstrated that strain 6N-NRC is a member of the genus *Bacillus* and is identified as *Bacillus aryabhattai* (Fig. 1). It is known that bacteria belonging to *Bacillus* produce a high PHA

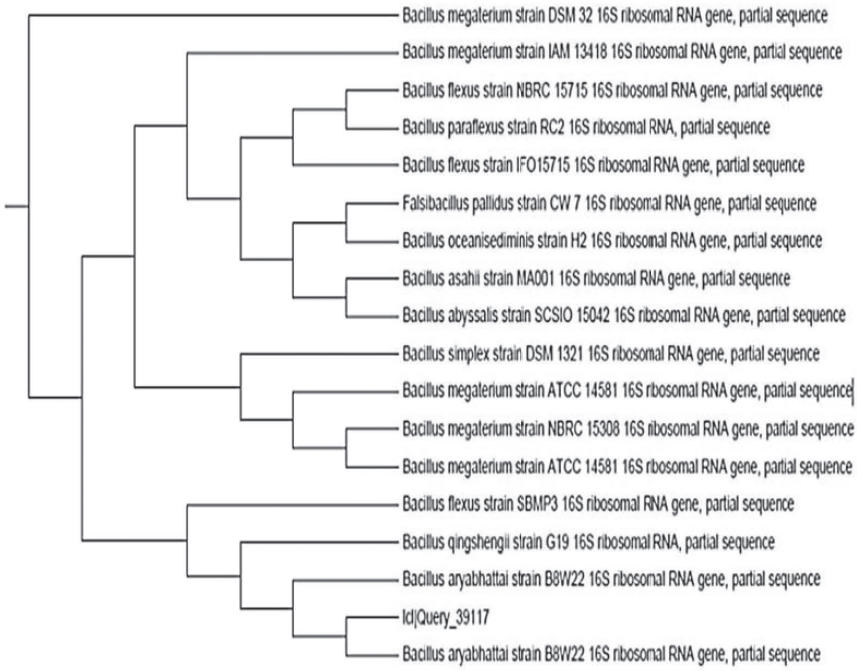

Fig. 1. Phylogenetic tree of the efficient *Bacillus* strain (6N-NRC) producing PHB in comparison to the most related bacterial strain *(Bacillus aryabhattai)* in database.

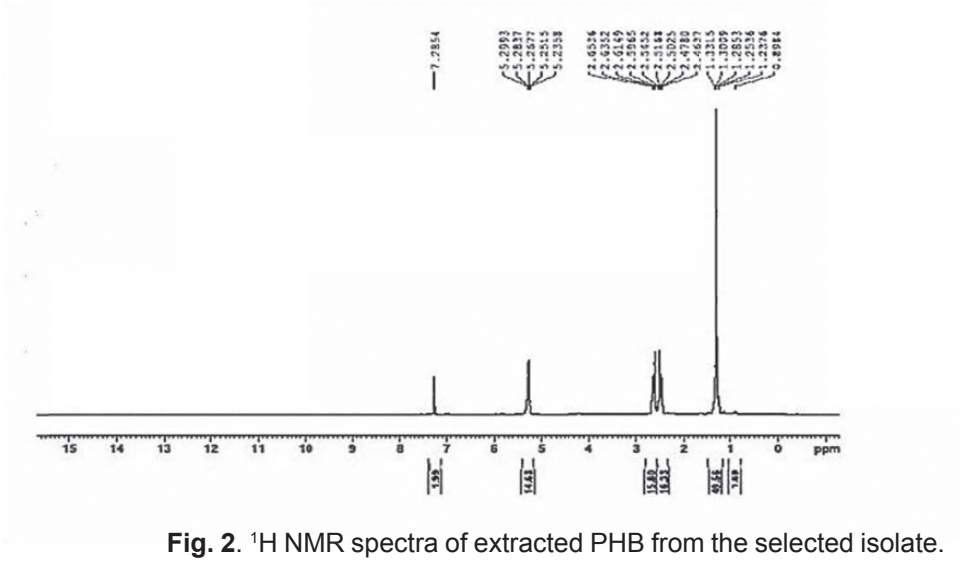

Fig. 2. ^{1}H NMR spectra of extracted PHB from the selected isolate.

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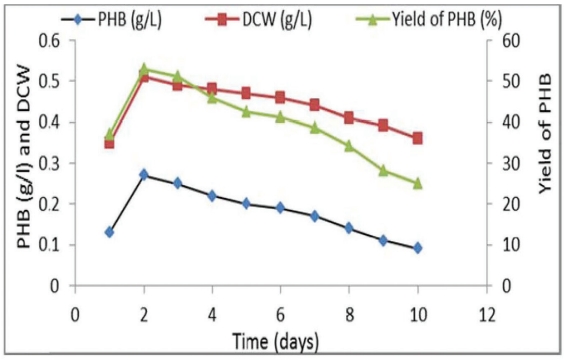

Fig. 3. Effect of incubation period on production of PHB using *Bacillus aryabhattai* 6N-NRC

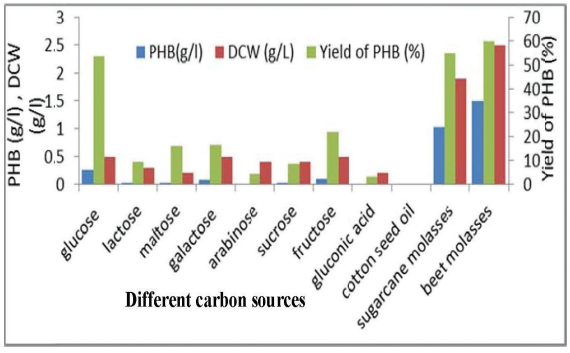

Fig. 4. Effect of different sources of carbon on production of PHB by *Bacillus aryabhattai* 6N-NRC

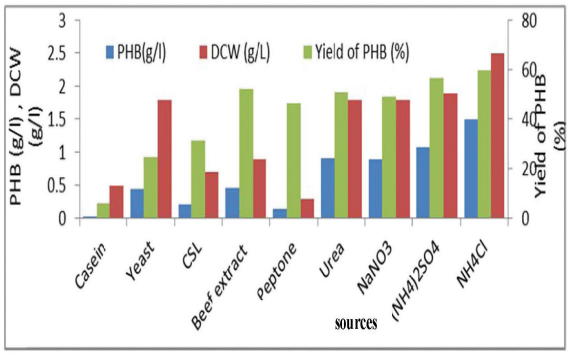

Fig. 5. Effect of different sources of nitrogen on production of PHB by *Bacillus aryabhattai* 6N-NRC

concentration [27, 28]. *Bacillus* sp. has the ability to synthesize both short and medium chain length PHAs (35).

Polymer analysis by ^{1}H-NMR spectroscopy : Based on NMR characterization of the PHA produced by selected bacterial isolate in comparison with the standard PHB (Sigma),it was found that the properties PHA produced are similar to that of the standard PHB(Sigma) (Fig. 2), so the PHA produced is polyhydroxybutyrate (PHB). The structure of polyester was investigated by ^{1}H NMR. The ^{1}H NMR spectra of the PHA extracted from selected strain show thefollowing resonance signals: HC=CH bond at 5.26 ppm ,CH_2O–COOH bond at 2.56 ppm, a high signal at 1.28 ppm that belongs to the hydrogen of methylene in the saturatedlateral chain, and a terminal –CH_3 group at 0.89 ppm (36).Three groups of signals characterizing PHB: a douplet at 1.28 ppm which is attributed to the methyl group, a doublet of quartet at 2.56 ppm which is attributed to methylene group and a multiplet at 5.26 ppm, which is characteristic of methine group. The ^{1}HNMR spectra of the sample and the standard are almost identical, which confirms that produced compound is polyhydroxybutyrate (PHB).

Optimization of culture medium for maximum PHB production: The nutritional, growth and physical factors such as the C-source, N-source, incubation time, pH and temperature greatly affect PHB accumulation (37). Therefore, these parameters were examined for maximum PHB production by the selected isolate.

Effect of incubation periods: The effect of time of incubation on production of PHBby the selected isolate was shown in Fig. 3. The optimum incubation period for the selected isolate was 48h where the PHB yield was 52.94% (w/w). This agrees with Hawas*et al*. (15), Valappil*et al*. (38), Kumar *et al*. (39) and Berekaa and Al Thawadi (40). On contrary,Pillai*et al*. [41] reported that *Bacillus aryabhattai*reached maximum polymer accumulation after 60 h. Other researchers found

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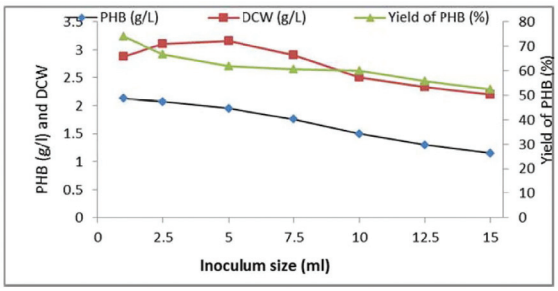

Fig. 6. Effect of inoculum size on production of PHB by *Bacillus aryabhattai* 6N-NRC

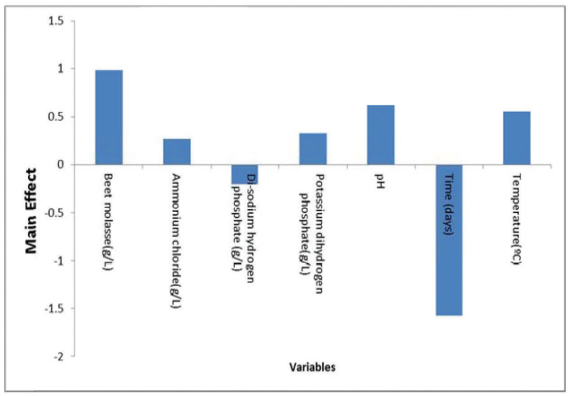

Fig. 7. Main effects of variables on PHB production by Bacillus aryabhattai

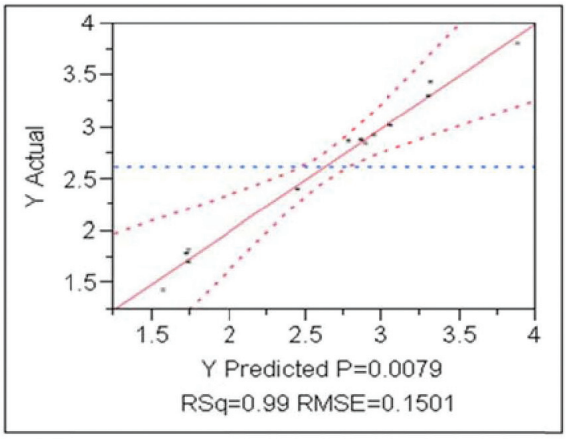

Fig. 8. Response Actual by Predicted plot of PHB production

that 24h was the best incubation period (42, 43, 44).

Previously, it was reported that bacteria which produce PHB were divided into two groups. The first groupincludes those which produce PHB during stationary phase when oxygen, magnesium, phosphorous and nitrogen are limited while the carbon source is present in great amounts. The second group involves PHB production in the growth phase (45). The Bacillus cereus belongs to the first group. In this study, the fermentation was for different incubation periods from 1 to 10 days. After 48h, maximum PHB accumulation was shown. This may be attributed to the acclimatization phase. Cell mass increased steadily, leading to maximum production at 48h followed by gradual decrease. This was due to presence of bacteria in its decline stage due to the decrease of nutrient supplements and accumulation of metabolites, toxins and inhibitors (38).

Effect of different sources of carbon : The effect of different sources of carbon (glucose, lactose, maltose, galactose, arabinose, sucrose, fructose, gluconic acid, cotton seed oil, sugarcaneand beet molasses) on PHB yield was shown in Fig. 4. Among the tested carbon sources, beet and sugarcane molasses were found to be the best carbon sources for PHB production with yield of 60%, 54.74%(w/w) respectively followed by glucose as a carbon source with PHB yield of (52.94% w/w). Beet molasses is an industrial waste and economic carbon source which leads to reduction of production cost. On the contrary, several workers found that simple sugars like glucose and glycerol are easily used by bacteria to enhance both growth and production of the polymer(46, 47). Carbon sources are important as they have three different functions within the microorganism which are: biomass synthesis, maintenance of the cell and PHA polymerization (7). One of the main problems for the extensive production PHBs is their high production cost. The selection of the suitable carbon substrate is considered as an important factor as it highly

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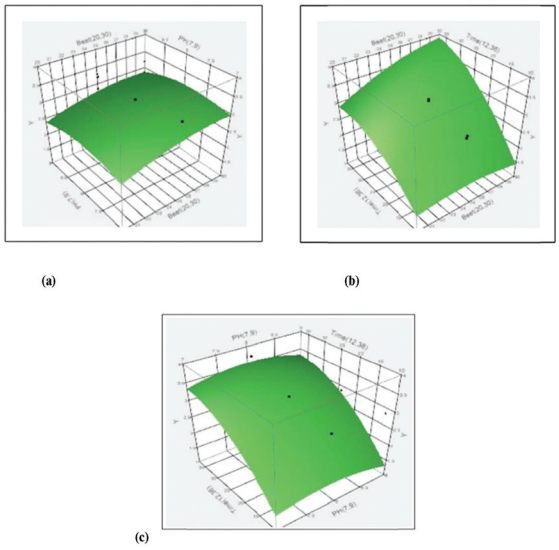

Fig. 9. (a-c): Three dimensional response surface curves revealing the effect of interactions of significant variables on production of PHB by *Bacillus aryabhattai* 6N-NRC (a) beet molasses concentration and pH (b) beet molasses concentration and time (c) pH and time.

affects the total cost of the produced PHB (48). Similar results were reported by Getachew and Woldesenbet (49)who obtained 56% PHB yield using pretreated sugarcane bagasse followed by corn cob with 52% yield. Yu *et al*. (50) showed that bagasse hydrolysate was used to produce 54% PHB yield.A strain of recombinant *E. coli* was reported to produce the polymer utilizingmolasses as source of carbon. The final dry cell weight, PHB content and productivity were 39.5 g/L, 80 wt% and 1 g/L/h, respectively (51).

Effect of different sources of nitrogen : To study the effect of nitrogen source for maximum PHB production, different organic and inorganic nitrogen sources were included in the MSM medium along with the best C-source (beet molasses), and the results are depicted in Fig. 5. Among all the tested nitrogen sources, NH_4Cl was found to be the best source of nitrogen which gave the highest yield of PHB (60%). This result agrees with Musa *et al*. (32) who reported that maximum PHB production was shown by NH_4Cl because it represents a readily utilizable nitrogen source using *Citrobacter*sp. and *Bacillus* sp.2. Species utilizing ammonium (NH_4^+) containing nitrogen source can be advantage with respect to industrial applications where in ammonia containing waste liquids can be utilized for the production of PHB (31). Mulchandani *et al.* (52) and Raje and Srivastava (53) by using *A. eutrophus* obtained highest PHB yield using ammoniumsulphate followed by ammonium chloride.

Effect of inoculum size: The inoculum size of the selected isolate was studied to stabilize intial microbial load 1% (v/v) was the best intial inoculum size where maximum PHB output was 73.96%. However, minimal PHB accumulation (52.27%) was achieved with 15 % (v/v) inoculum size (Fig.6). This may be attributed to the fact that a small size of inoculum may results in a number of microbial cells which is insufficient and therefore a low amount of secreted enzymes, On the contrary larger size of inoculum may cause oxygen reduction and nutrients depletion in the fermentation media (54).

Determination of fermentation factors affecting PHB production using Plackett-Burman design: To reduce the production cost and maximize the yield it is important to optimize the fermentation medium and conditions. The recent optimization studies have depended on statistical experimental design and response surface analysis. Statistical design is an efficient method to explain the major fermentation parameters and also their interactive effects on the process. It is an effective way to identify significant variables effective on PHB production, thus minimize the process development time and cost (55). Plackett-Burman design offers good and quick screening method and mathematically computes the significance of large number of factors in one experiment, which is time saving and maintain convincing information on each component (56). The design is recommended

when more than five factors are under investigation (57).

Screening of the most effective factors affecting PHB production was done using the Plackett-Burman design. Seven components were chosen for the study where each variable was tested at two levels, high concentration (+) and low concentration (-) in 8 trials and all the experiments were done in triplicate, and average of the results was used as the design response (Table 3). The variation in PHB production was ranging from 0.164 to 3.252 g/l. This showed that these parameters have a strong effect on production of PHB. The maximum production of PHB was observed in trial ordered 4 where the production of PHB and percentage yield of PHB increased to 3.252g/L and 79.07% respectively.

Analysis of the effect of the physicochemical parameters of the media showed that the production of PHB is highly affected by time, beet molasses concentration and pH. On the other hand, concentration of NH_4Cl, KH_2PO_4 and Na_2HPO_4 and temperature adjustment had relatively lower effect (Fig. 7). Many investigations showed that both pH and incubation time had significant influence on PHB production (58, 59, 60). However, other reports pointed critical importance of carbon source on PHB production (61, 62).

Depending on Plackett-Burman experiment results a pre-optimized medium composed of: KH_2PO_4 1.5g; Na_2HPO_4 3.525g; $MgSO_4.7H_2O$ 0.2g; $CaCl_2$ 0.02g; NH_4Cl 0.75g; ferric citrate 0.0015g; trace elements solution 1ml; distilled water 1000 ml inoculated with inoculum size 1% (v/v) of 48 h old culture incubated on rotary shaker was used in response surface methodology (RSM) for further conditions optimization.

Response surface methodology: The 3 variables (Beet molasses concentration , pH and time) identified by Placket-Burman design experiment having higher main effect on PHB production were further tested and optimized through Box-Behnken design methodology . This method allows the interaction of three independent variables at three different levels low (-1), medium (0), high (+1)) were listed with 13 trials (Table 4)

Box – Behnken design of RSM was used to estimate the influence of each individual factor and also their interaction effects on PHB production by the selected isolate. Response surface analysis showed that beet concentration 30 g/l, pH value 8 and an incubation time of 36 hours were the optimum production conditions. Other variables were kept in their (zero) initial values. To validate the exact optimum values of Beet molasses, pH and time and their interactions, statistical designs were used and the ranges between the optimum points were selected. Therefore, Box-Behnken design was focused on the interaction between these three factors while other factors were effectively insignificant for PHB production. In this connection, optimization of the effective factors on production of the polymer and their interactions were tested by Lakshman*et al*. (63) and Prabisha *et al*. (60), Box-Behnken design was applied to detect the optimum level of each factor. Design-Expert software was used to design Box-Behnken matrix and PHB yields of all trials are given in Table 5. Multiple regression analysis was used to analyze the experimental results and the second-order polynomial equation was formed to explicate the PHB yield through quadratic equation as follows:

$$Y = 3.0516222 + 0.2446023(X_1) - 0.043696(X_2) + 0.8283713(X_3) - 0.000305(X_1)(X_2) + 0.2515275(X_1)(X_3) + 0.0348075(X_2)(X_3) - 0.164019(X_1)^2 - 0.233841(X_2)^2 - 0.337969(X_3)^2$$

Where Y is the response (PHB yield), X_1, X_2 and X_3 are terms coding for the variables including beet molasses concentration, pH and incubation time respectively. The equation was found to be statistically significant by evaluation using F-test for analysis of variance, which shows that the regression analysisat 98% confidence level ($P<0.05$) is highly significant as shown in Table 5 and 6. ANOVA for yield of PHB showed that F value of 32.0101 with a low probability value and

P-value of the quadratic regression model (P>F 0.0079) which proves the strong significance of the model. The R^2 value of 0.989 (so near to1) which confirms the model accuracy showed a strong correlation between the actual and the predicted values of PHB yield. Therefore, the model fits 98.9% of the total variations in PHB yield. The optimum values of the various variables differ according to the microorganisms selected for production of PHB and their place of isolation and in turn alter their physiological characteristics (64). The Actual by Predicted plot provides a visual assessment of model fit that reflects variation due to random effects. Fig. 8 plots the actual values of response (PHB production) against its marginal predicted values.

To determine the optimum values of beet molasses concentration, pH and incubation time for optimum yield of PHB and studying their interactions, three dimensional response surface curves were plotted as illustrated in Fig. 9(a, b, c). Fig. 9(a) shows that the increase in pH and beet molasses concentration upto 8 and 30 g/L respectively increased the yield of PHB. On the

Table 1. Isolation and screening of PHB producing isolates

Soil sample	Total No. of different types of isolates	No. of PHB positive isolates (producer)	No. of PHB negative isolates (non-producer)
Soil A	7	3 (A1toA3)	4
Soil E	8	2(E1,E2)	6
Soil G	5	3(G1,G4,G5)	2
Soil J	4	1(J3)	3
Soil K	6	2(K2,K5)	4
Soil N	8	4(N1 to N3, N6)	4

Table 2. Biomass and PHB production by 15 positive bacterial isolates obtained from different soil samples

S.no.	Soil sample	PHB positive	PHB (g/L) isolate	Biomass (g/L)	PHB yield (%)
1	A	1	0.092	0.6815	13.5
2		2	0.109	0.6813	16
3		3	0.158	0.79	20
4	E	1	0.149	0.582	25.6
5		2	0.163	0.519	31.4
6	G	1	0.152	0.527	28.8
7		4	0.115	0.6534	17.6
8		5	0.126	0.689	18.3
9	J	3	0.098	0.624	15.7
10	K	2	0.135	0.572	23.6
11		5	0.086	0.672	12.8
12	N	1	0.160	0.575	27.8
13		2	0.122	0.589	20.7
14		3	0.148	0.655	22.6
15		6	0.17	0.44	38.63

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Table 3. Nine trial Plackett-Burman design matrix for seven variables predicted for PHB production *Bacillus aryabhattai*

Trial no	Beet molasses (g/L)	Ammonium chloride (NH_4Cl) (g/L)	Disodium hydrogen phosphate (Na_2HPO_4) (g/L)	Potassium dihydrogen phosphate (KH_2PO_4) (g/L)	pH	Time (days)	Temperature (°C)	Production (g/L)	Dry weight (g)	Yield (%)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7			
1	-(15)	- (0.5)	-(2)	+(2)	+(8.5)	+(3)	-(25)	0.26439533	2.302	11.485
2	+(25)	-(0.5)	-(2)	-(1)	-(6.5)	+(3)	-(25)	0.98512974	3.807	25.877
3	-(15)	+(1)	-(2)	-(1)	-(6.5)	-(1)	+(35)	1.68814559	3.182	53.053
4	+(25)	+(1)	-(2)	+(2)	+(8.5)	-(1)	- (25)	3.25168513	4.112	79.078
5	-(15)	-(0.5)	+(5)	+(2)	-(6.5)	-(1)	+(35)	1.7011752	3.301	51.535
6	+(25)	-(0.5)	+(5)	-(1)	+(8.5)	-(1)	+ (35)	2.28684118	3.012	75.924
7	-(15)	+(1)	+(5)	-(1)	-(6.5)	+(3)	- (25)	0.16370211	2.34	6.996
8	+(25)	+(1)	+(5)	+(2)	+(8.5)	+(3)	+(35)	1.21428571	3.816	31.821
9*	20	0.75	3.525	1.5	7.5	2	30	2.13	2.88	73.960

Table 4. Box-Behnken design of significant variables affecting production of PHB by Bacillus aryabhattai

Trial	Independent Variables			Yield (g/L)
	Beet (X1)	PH (X2)	Time(X3)	
1	(-) 20	(-) 7	(0) 24	2.396
2	(+) 30	(-) 7	(0) 24	2.919
3	(-) 20	(+) 9	(0) 24	2.868
4	(+) 30	(+) 9	(0) 24	2.888
5	(-) 20	(0) 8	(-) 12	1.826
6	(+) 30	(0) 8	(-) 12	1.779
7	(-) 20	(0) 8	(+) 36	2.840
8	(+) 30	(0) 8	(+) 36	3.799
9	(0) 25	(-) 7	(-) 12	1.701
10	(0) 25	(+) 9	(-) 12	1.439
11	(0) 25	(-) 7	(+) 36	3.427
12	(0) 25	(+) 9	(+) 36	3.306
13	(0) 25	(0) 8	(0) 24	3.005

contrary, the more increase or decrease in these two values lowered PHB production. Fig. 9(b) shows the interaction between beet molasses concentration and time, which depicts the highest beet concentration at incubation time of 36h whereas any variation of these values decreased the produced PHB. Fig. 9(c) shows the interactive effect of time of incubation and pH. Thus the best values of the tested variables were beet molasses concentration 30g/L, pH 8 and incubation time 36h to obtain 3.7992 g/L of PHB. Hassan *et al*. (64) reported the great influence of time of incubation on PHB production using *Bacillus subtilis*.Gouda*et al*. (65) found that both glucose and molasses used as sources of carbon have strong effects on biopolymer production. This may be attributed to the fact that PHB production is related to the high concentration of carbon source as well as molasses is considered a mixture of different salts as mineral source. This is comparable to maximum PHB production 5.41 g/L obtained by using molasses as source of carbon by *B. megaterium* in batch culture (66, 67).

Conclusion

A new bacterial strain which could produce PHB was isolated from Egyptian soil and was identified by using 16S rDNA technique as *Bacillus aryabhattai*. Response Surface Methodology approach through Box-Behnken design for optimization of synthesis of PHB showed high efficiency to increase PHB yield. The results indicated the impact of beet molasses concentration and the time of incubation on PHB production which is an economically and environmentally important product and can solve

Table 5. Analysis of variance

Source	DF (Degree of Freedom)	SS (Sum of Squares)	Mean Square	(R squared) R2	Adjusted R squared	F Ratio	Prob > F
Model	9	6.4884444	0.720938	0.989694	0.958776	32.0101	0.0079*

Table 6. Results for ANOVA analysis for optimization of PHB production by *Bacillus aryabhattai*

Term	Coefficient Estimate	Std Error	t ratio	P-value
Intercept	3.0516222	0.13934	21.90	0.0002*
X1 (Beet)	0.2446023	0.063472	3.85	0.0309*
X2 (pH)	-0.043696	0.058705	-0.74	0.5107
X3 (Time)	0.8283713	0.053059	15.61	0.0006*
X1X2	-0.000305	0.102394	-0.00	0.9978
X1X3	0.2515275	0.075037	3.35	0.0440*
X2X3	0.0348075	0.075037	0.46	0.6743
X12	-0.164019	0.10404	-1.58	0.2130
X22	-0.233841	0.105889	-2.21	0.1143
X32	-0.337969	0.094248	-3.59	0.0371*

one of the problems of the environment which is waste management. Using byproducts such as molasses reduces the production cost. Moreover, molecular approaches are recommended to improve microbial production of PHB.

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A New Approach for Tracing Adulteration of Saffron with Safflower by Universal Barcoding Primers

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Abstract

Saffron adulteration dates back to antiquity a fraudulent act continued to modern day. In recent years the wide use of saffron in medicine and food has been associated with wider use of counterfeit to saffron, nevertheless this has been associated with development of methods to detect such adulteration.

The advancement of molecular biology methods has been one of key area used for detection of adulterants. The concept by its own is diverse and different approaches for better detection of adulterant has been developed to support detection of fraudulent activities in particular in food and agriculture industry.

To develop primers for detection of saffron adulteration with safflower we designed primer pairs based on universal reference sequence for chloroplast trnl(UUA) Intron that are different in length and size for saffron and sawfflower.

The approach was to save tedious work using otherwise RAPD /SCAR (sequenced characterized amplified region)markers to have accurate reproducible results with least interference and higher level of polymorphism of particular and specific conserved region in plants.

Key words: Saffron, the *Crocus sativus,* adulteration, safflower.

Introduction

Saffron exact origin is a matter of controversy, but most certain has originated from Iran, Greece and later spread over to Mediterian, India, and china. (1, 2). Saffron is a triploid,male-sterile species not known to be wild or spontaneous (3). It enjoys certain, agronomic, eco-physiological features including a relatively low water use, growth and development during fall and winter. It has very low harvest index as its cultivation, collection and handling practices, calls for careful and special attention, making it a direct and high labor and most precious agriculture commodity (4, 5). Saffron precious value should be sought in its limited production and wide spread application. Saffron is mainly used in food and pharmaceutical industries, two life-dependent and ever expanding markets although,textile industries too, does exploits saffron as a dye nevertheless it is used even in perfumery(6).

Saffron limited resources, and increasing demand, commands its high value and does justify the inexcusable alternative to compensate its short supply with deliberate substitution or adulteration with other materials. Saffron is known for its color, aroma and flavor providing grounds for easiest fraudulent activity which is use of synthetic dyes to increase the coloring strength of its aqueous extract, an act in violation of rules and regulations in most of the countries .The other fraudulent activities include substitution of other

plants with saffron, such as *Carthamustinctorious* or safflower, *Calendula officinalis* or marigold, arnica and tinted grasses,besides mixing of beet and pomegranate fibers,are other frequent adulterants. The other practice in saffron fraudulent activities is to increase product mass by mixing yellow stamens of saffron with saffron stigma or powder, nevertheless amongst all these, most common fraudulent activity is substituting safflower petals with saffron stigmas (7).

There are various methods used for detection of adulterants in saffron, ranging from physical and microscopic examinations to spectroscopy method, in addition to high performance liquid chromatography which enjoy high precision and accuracy.The coloring property of saffron resulted from its various pigments has been applied in chromatography and spectroscopy methods for detection of some adulterants in saffron. Although the detection and sensitivity of HPLC method makes it the most accurate and acceptable method for detection of adulterants, the cost of methods compared to other methods are quite high.(6)

The international ISO-standard 3632-2 and its technical specification ISO/TS 3632 covers procedures for all the above methods which specify and define saffron taste, fragrance,color, moisture, minerals, exogenous dyes and foreign materials for authenticity and grading of saffron. Recent publications and comments over this ISO standard,highlights weak reliability of some of the mentioned methods and high cost of reliable methods to an extent that some of the researchers have suggested use of different methods for same sample to increase the reliability of results(8).

Advancement of molecular biology and DNA based techniques have initiated new approaches for detection of adulterants specifically for agriculture commodities.The advantages associated with DNA based techniques are important because of their application to all living organism(9).Unique genetic composition of each organism and their consistency and robustness in various physiological and environmental conditions makes them the most reliable elements for identification of organisms. Methods based on genetic materials have solved the problems made by age, degradation, and storage conditions of samples (9, 10).

The DNA basedtechniques could be classified into three types, namely polymerase chain reaction (PCR), sequencing and hybridization based, while the most robust, simple, sensitive, specific, rapid of all them is polymerase chain reaction (PCR), Which requires inexpensive markers, making it most cost-effective among DNA based methods compared to instrumented based methods, it is quite accurate,and accepted as an economical approach in food authentication (9).

The polymerase chain reaction amplifies specific DNA regions directed by oligonucleotide primers into easily detectable level, specifically in case of agricultural products.The main question in PCR reaction is how well a DNA region is characterized to serve the purpose of reaction and how accurate, specific and sensitive that region is reproduced along with oligonucleotide primers. Various PCR-based methods used for adulterants detection and authentication have been developed to increase the quality of detectionas well as the specificity and sensitivity of method reaction including PCR with arbitory primers (AP-PCR), PCR-RFLP (restriction fragment length polymorphism), RAPD and its modified improved version, SCAR marker, but the core issue has yet remained that how best and accuratein shortest time period particular region amplification is achieved. (1, 11, 12).

To initiate a DNA based method for identification of species, DNA based barcoding concept was developedfar beyond morphological classification in taxonomy.The application of barcodingis quite wide in different fields, forensic science, biotechnology, food industry, animal diet and many other areas.This concept uses standardized DNA region in specific loci as a tagfor species identification.DNA barcoding as an identification technique enjoys several advantages,

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including feasibility of performances even with degraded material, low DNA requirements, simplicity, rapidness, time and cost effective protocols, in addition to reproducible results between laboratories(13).

In wake of economic globalization and new trade approaches, authentication of food and its related products for food safety has been one of the most debated issues in recent years. Increasing the usage of agricultural products with dual application both as food and herbal medicine has strengthened concerns over food authenticity and safety. This has been one of the prime reason for exponential research in food analysis, an indispensable tool for authentication of food and food products, particularly in case of food which have dual or numerous biological applications like saffron (14, 15).

To develop an accurate, specific, primer sequence for authentication of saffron free of safflower in a time and cost efficient way, we used an international recommended standard sequence of chloroplast trnL (UAA) intron as template. This standard sequence has been recommended in ISO standard 21569 for plant authentication from other biological samples. The difference of nucleotide sequence in trnL (UAA) intron region of saffron and safflower implicated in their length was used to design primer sequence for each. These primer sequences were used to trace safflower if mixed with saffron by their difference in length sequence.

Material and Methods:

Plant materials: To ensure pure saffron sample for genomic DNA extraction ,we collected sample directly from harvesting site in Qanat city of Khorsan province with certificate of analysis for approval of its authenticity by HPLC. For safflower we collected the flower from Agriculture research center in Karaj of Alborz Province.

DNA extraction: Total genomic DNA was isolated using CTAB protocol of ISO standard 21571.

Designing of Specific Primers: For differentiation of saffron with safflower Multiplication of chloroplast trnl gene (UAA) intron.

To design specific primers for tracing adulteration of saffron with safflower samples of both plants were amplified with specific region of chloroplast trnl gene (UAA) intron referred in Gene Bank with accession No. Z00044, X1590 and referred in ISO standard 21589 whose amplification protocols has been specified in Table-1.The resultant PCR reaction was a 500 bp product as shown in Fig. 1 and expected by the said protocol of ISO 21589.

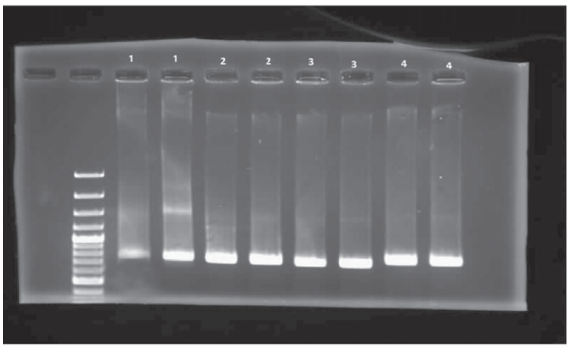

Fig. 1. The result of PCR products of universal trnl gene (UAA) Intron in four different Plants namely Pistachio, Favabean, safflower, Saffron having 500 bp nucleotide sequence.

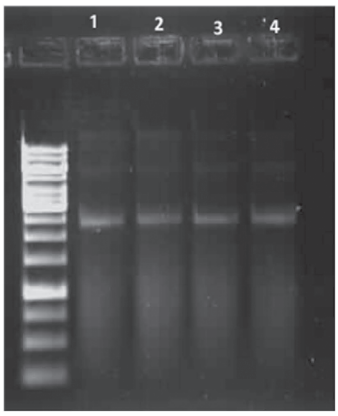

Fig. 2. Plasmid extraction.

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DNA Sequencing : Amplified PCR products were separated electrophoretically in 2% agarose gel, excised from the gel,and purified with IBRC extraction kit (MBK0061) and cloned with PGEM-T easy Vector (promega Crop, Madison, WI, USA). The transformed bacterial colonies were screened. Two colonies were cultured in LB medium and thereafter plasmid extraction was done with the help of IBRC kit seen in the Fig. 2 and extracted colonies were sent for sequence analysis to GATC company in Germany.

Primer design and optimization of PCR conditions: Specific primers for identification of saffron and safflower were designed according to nucleotide based difference between the two amplified trnl PCR products using programs, bio-Edit and prime blast. The primer sequence and their amplicon length' (Fragment size) are given in table 2.

Results

To evaluate the application of designed primers their validation was necessary. The validation of primers designed had to be sought in its properties and ability to trace safflower in saffron packing.

Validation of designed primers: A multiplex PCR program whose protocol is given in table-3 was set up to evaluate species specificity performance of primers and to rule out cross reaction with most common plants.

The target sequences are found in the plants chloroplasts and no sequence similarity should be seen in non-plants organisms,so amplification of both primer pairs were done with DNA extracted from human blood.

The primers were designed to amplify Crocus Sativus. (saffron) and C. Officinalis (safflower). To rule out cross reaction of the designed primer pairs with other plants DNA these primer pair were amplified with pistachio,corn, walnut and pepper. As seen in the Fig. 3 there were no cross reaction between primer pairs with DNA of the selected plants.

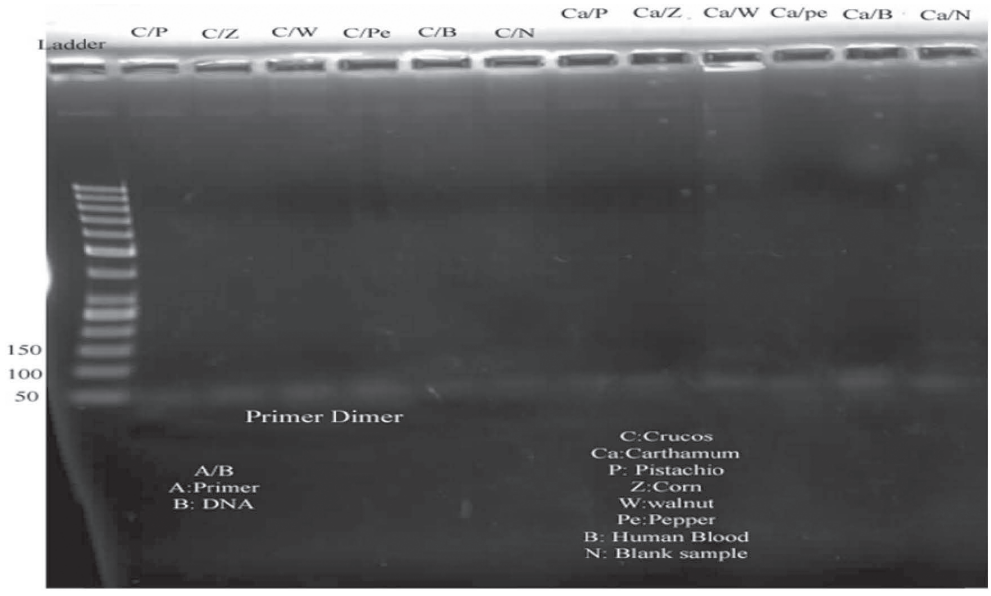

Fig. 3 : Evaluation of cross reaction of both of the designed primers with other plants DNA and non related Species DNA.

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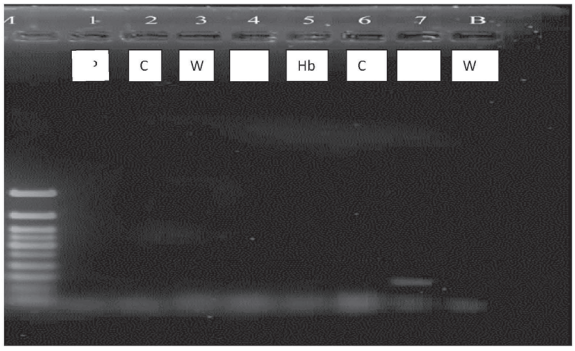
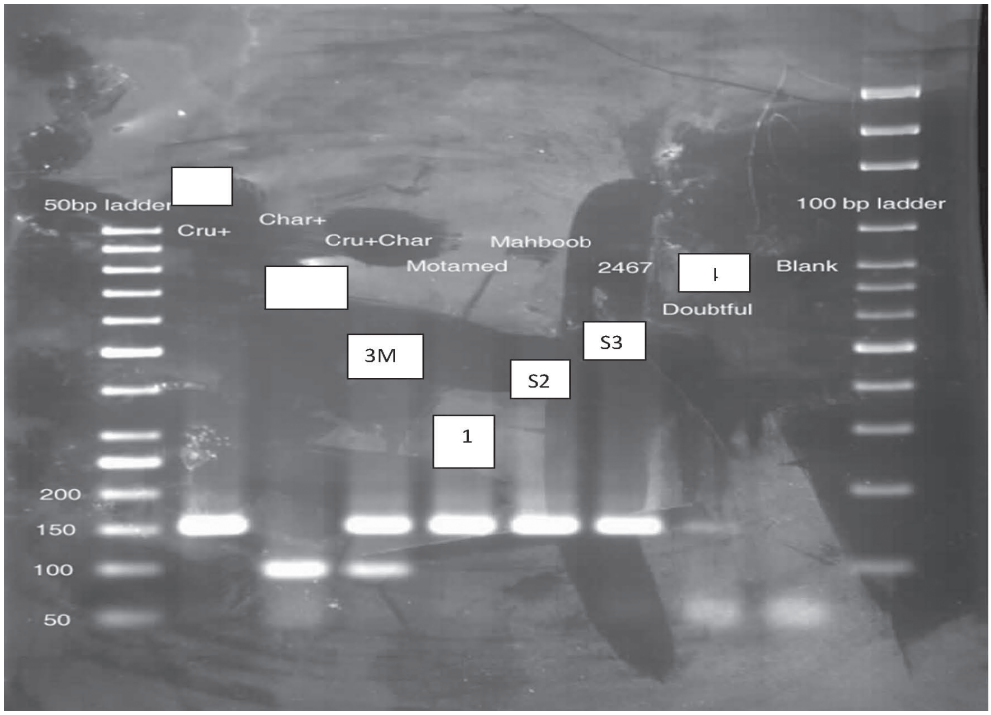


Fig. 4. Validation of primers specifity to rule out common adulterant interference of saffron along with other plants DNA. P- Pistacho C- Corn W-Walnut PE-Peper Hb-Human Blood C- Calendula officinalis T-Tumeric Powder W-water

As the difference of amplicon length was a discriminatory factor in different plants, the DNAs extracted from, pistachio, corn, marigold, pepper, human blood, and turmeric powder were amplified by the designed primers in order to evaluate the specificity of primers by the expected length of PCR product as shown in image 4. As shown in image 4 other than turmeric powder non of the primers gave band with the sample and the band for turmeric powder was in the vicinity of that of saffron but not in the same location.

To validate application of primer pair for tracing adulteration of saffron with safflower. We analyzed the designed primer reaction with commercial packed samples from the market. The image-5 shows the results. As Shown in



Fgi. 5. A market survey results for primer and PCR protocol confirmatory ability to trace adulteration of saffron. 1S- Pure Saffron sample 2F-Pure Safflower 3M-Mix sample of saffron and safflower .-S1sample one with trade name Motamed S2 sample with trade name Mahboob.S3-Unknown trade name sample with laboratory identification code.S4-Dyed inferior Saffron Sample.

image samples have been designated with S and differentiated with numbers. The most important sample was 4th sample as it was saffron which had been dyed with edible colors to compensate for inferior quality of saffron packed where its PCR product was faint but enough to trace the sample as saffron.

Discussion

In this research we developed two pairs of primers from a conserved well recognized region in chloroplast (trnl),in order to detect safflower as an adulterant in saffron samples. The trnl region in chloroplast which is unique gene, in the plants, has been introduced in ISO standard 21569 as a reference sequence for confirmation of DNAs extracted from plants which has been validated and documented the oretically and experimentally by testing in various samples of plants and non-plants. However, we did trace and found a difference in the length of the mentioned sequence between safflower and saffron, which was used for detection of safflower in saffron as an adulterant.

The international standards for saffron (ISO/TS 3632) consist of two parts.The standard has been drafted and approved by ISO technical committee ISO/TC 34 SC7 spices and condiments. The second part (ISO/TS 3632-2) covers test methods, for evaluation of quality and authenticity of saffron.The standard covers macroscopic and microscopic examinations as well as physical and chemical testused to specify the quality of saffron.

As number of reviews and research papers have indicated the chromatographic and spectroscopy methods have limitation in detection of adulterants since phytochemical in plants species varises with growing conditions harvesting periods, post harvest process and storage conditions a reason which could be misleading results if the sample has been adulterated with same adulterant compound (15, 16).

The UV-visible spectro photometric test methods such as TLC and HPLC,are used to characterize the phytochemical properties of saffron similar to many other standard test methods for authentication of medicinal plants. Sabestinia and etals have indicated that UV-Visible spectrophotometric method could not specify nature and type of adulterants,and it has some limitations to distinguishing blended adulterants with saffron below a certain w/w percentage.The adulterants could not be specified clearly by the TLC procedure stated in ISO/TS 3632-2 which has been developed based on the phytochemical specifications, so false results may be obtained by this method affected by age or storage conditions of samples. HPLC method has been designated by researchers as the most accurate method due to its ability to trace most colors used as adulterants of saffron due to their appearance and coloring properties. Sabestinia and etals have pointed out that HPLC/PDA/MS technique allows the unequivocal identification of adulterant characteristic marker molecules based on the values of absorbance and mass. Even though HPLC/PDA/MS method has been praised for accuracy and precision for detection of adulterants, it has been realized that this method is not easily available because of to its equipment basis and expensive instrumentation not preferable for field activities.

Although chemical methods based on phytochemical properties of saffron and spectro photometric absorption properties of additive colors provide useful clues for tracing adulterants, they can not provide sufficient and strong evidences to identify the type of the adulterants. (Anna Torelli and etals). Moreover, chemical finger printing could be influenced by various factors including age of sample, physiological conditions, environmental factors, cultivation area, harvesting period, drying and storage conditions (9).

The advancement of molecular biology and its rapid expansion and improvements has revolutionized many scientific fields including food safety and authenticity.The DNA-based techniques have been extensively used in food authentication for their specificity, time and cost efficiency (9) (6)..

As DNA is extremely resistant, stable and long lived macromolecule that even its slightest amount can be recovered from any fresh, dried, and even processed material hence the techniques based on DNA are found ideal for molecular species identification with wider application even in food authentication as food are agro or animal based. Required properties of ideal DNA markers includes easy availability, highly polymorphic and reproducible, codominant inheritance and recurrent occurence in genome,selectively neutral to environmental conditions and easily applicable between laboratories (16). The DNA based techniques when compared to each other do differ for their analytical ability such as discriminatory power, sensitivity, reproducibility, cost and time efficiency besides their user friendliness. (5, 9, 11, 17).

Filipe Pereira reported that DNA-based hybridization techniques other than its high cost is not suitable for mixture detection due to cross-hybridization of closely related species nevertheless it is not even a time efficient method and requires good quality of DNA.The PCR based molecular techniques has been regarded as convenient for molecular studies as it facilitates any genomic region amplification providing genetic information of many individuals without requirements of cloning and isolating large amounts of ultra pure genomic DNA (18). This does not make PCR based method free of defects. Reports and reviews on PCR-based molecular DNA techniques like RAPD, ALFP and their improved versions like SCAR have high lighted the main difference revolving each PCR based techniques, including ,the requirement over quantity and quality of DNA,level of polymorphism, technical and instrumental demand and necessity for prior sequence information for producing reproducible results with affirmative decisive resolution free of interferences due to homology of similar size or limited size of amplified fragements for specific genetic loci(10, 12, 15, 18).These are implicit of two important issues lack of standardization and universtality(5).

With advancement of molecular biology and Herbert proposal, DNA barcoding has been used as a tool for species identification beyond single species. There is long way to reach a consensus on its application as a unique and most reliable method but it could be called a novel molecular and bioinformatical tool designed to provide rapid, accurate, automatable, cost effective method using a standardized DNA region as tag.The tag or standardized region is the solution of main issues associated with single species specificity of DNA-based techniques.The DNA based authentication assays require polymorphic and high copied, analytical target regions which also should be less variable within,than between species calling for conserved priming sites to make it extremely robust,and highly reliable DNA sequence and amplicon (5, 19).

Chloroplast DNA with its unique circular, small genomic size and conserved structure is one of the best candidates for plant barcoding. The chloroplast nucleotide substitution rate is far greater when compared to plant mitochondria DNA even though their genome size and arrangement vary enormously (20, 21). Moreover the chloroplast DNA circular structure could be divided into number of small and large repeat regions with number of loci which are used in barcoding studies.The most common loci which have been used for barcoding include rbcL, trnL-trnF, atpB-rbcL, trnLintron, matK, trnT-trnL (9, 13, 21).The plastid region of trnl (UAA) intron have been used in several studies discriminatings several plant genera and species. The trnL (UAA) intron region has unique evolutionary characteristic which gives it conserved secondary structure with alteration of conserved and variable regions (22).

The primer pair used in current study has been referred in the ISO 21589 and also one of the four primers referred by Taberlet.P in his studies, which encompasses the entire trnL (UAA) gene plus a few base pairs on each side belonging to trnL (UAA) itself. Taberlet. P has indicated the length of sequence amplified with c and d to vary from 254 to 767 (22). These primer sequence are

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extremely conserved in particular among angiosperms, and is most universality known sequence among plants which explains its robustness of amplification process that is a necessity for standardization of tag region (23).

As mentioned earlier in developing RAPD primers, the primer sequence is arbitary and no prior sequence information is required,the primers are cheap and process needs no blotting and hybridization, resulting in quick and simple matched stretches of DNA. The draw back of technique involves over results of amplified stretches of nucleotide which are mixture of various size and does not specify a specific loci. In addition these amplified nucleotide sequence are totally dependent over purity,quality, and molecular weight of DNA nevertheless PCR cycling conditions also does influence final product amplification resulting in total absence or decreased amount of banding profile of unspecific loci. This makes the RAPD results quite unreliable for decisive interpretation in authentication between two different biological product (11, 12, 24).

The modified version of RAPD is SCAR (sequenced characterized amplified region) the marker prepared through knowledge provided by RAPD or other alternative is to reduce repeatability problem and tedious procedure by means of primers designed from nucleotide sequence established in cloned RAPF fragments linked to a particular loci converting dominant markers into co-dominant markers. SCAR markers benefit from longer primer sequence which impart specifity, and higher level of polymorphism because of higher annealing temperature but as specified known standardized loci information is not used you cannot have information about trait of interest (10, 12, 16).

We exploited a short standard primer sequence known to amplify a conserved loci of

Tabel-1: PCR Temperature-time program for amplification of chloroplast trnl gene

The amplification Temperature-time protocol	
Activation/initial denaturation	4 min/94 °C
Amplification	30 s/95 °C 30 s/55 °C 120 s/72°C
Number of cycles	35
Final extension	5 min/72 °C

Table-2. The primer sequence of trnl PCR products of saffron and safflower used for differentiation

Marker	Sequence	No. of Nucleotide	Fragement Size
Carthamus	F: CAAAGGTTCAGAAAGCGAAAATCA R: TCTACCAACGTAAGACAATCAAC	24 22	94
Crocus	F: TTGACTACGTTGTGTTGGTAGCC R: CCACAATAACTCCCCCTTTTG	23 21	147

Table -3 Multiplex PCR protocol for amplification of designed modified trnL primers.

Activation/initial denaturation	4min/94°C
Amplification	30s/95°C 30s/55°C 120s/72°C
Number of Cycles	35
Final Extension	5min/72°C

chloroplast DNA used primarily for plant barcoding to facilitate DNA extraction and amplification and establish a stable assay protocol to trace a single adulterant resulting in less labors, less tedious, and cost effective procedure to design a specific primer as compared to RAPD and its SCAR version.

We have used a sequence of universal primer pair for amplification of trnL (UAA) intron a known conserved chloroplast region of plants with significant sequence information available at database and known to be extremely well conserved among angiosperms. These are fundamental characteristic for a universal and robust primer. Robustness, of a primer signifies amplification of conserved and well documented region which could define a standardized region for a standardized protocol for identification and tracing different species and genus(22).

The trnL (UAA) intron region have been used in simple PCR approach to trace and identify specific food crops and food allergens, more advance approach for identification and authentication has been in combination with lab-on-based chip capillary electrophoresis system for tracing olive oil, coffee, and wines adulteration. The difference in trnL amlpicon target length/ (PCR fragements lenght) and or in combination,if recognized selected SNP position due to allelic variants, were used as discriminatory measures (25, 26).

The molecular methods for tracing safflower adulteration (6) in saffron or for phylogenic studies usually have focused on using RAPD/SCAR markers including Javanmardi.N and etal, Beiki H. A andetal, Marieschi.M, and etal, Gaiol.G and etalneverthless methods like ISSP primers has been use of by Han-jieZheng and et al (1, 6, 7, 27-29).

We designed set of primers from already well documented, conserved, standardized, known, polymorphic and high copy analytical targets, whereas other studies did have to trace and find such region even though then also the position of loci could have not been proved to be standardized neither its robustness nor its replication rate. We did avoid time consuming procedure of RAPD/SCAR and other molecular markers identification procedures.

The evaluation criteria for specificity of primers to distinguish between saffron and safflower did prove primers ability to detect safflower both in samples made in laboratory for evaluation of primers and also samples collected from market. Validation of designed primers showed no cross reaction with non-plant DNAs .

Comparing the length of PCR products amplified by saffron, sawflower, marigold, pistachio, corn and pepper showed that the designed primers are quite specific to saffron and sawflower according to their length. The PCR method was validated for possible cross reaction leading to conflicting results. There were no cross reaction other than with turmic powder, though band was not formed at the same location as that of saffron, it was in the vicinity of saffron band, but due to the aromatic characteristics of turmic powder, it would not be used as an adulterant in saffron packing restricting application of these primer for tracing saffron adulterants only for saffron packs.

In conclusion we could exploit successfully a universal conserved and standardized DNA trnL loci in chloroplast gene to design set of primers unique for plants and specific to saffron and sawflower differing in length of their PCR products

to distinguish sawflower from saffron as an adulterant. The development of primers took a very short time with acceptable accuracy, robustness and cost effective approach. Considering wealth of various plastids DNA regions proposed and recommended to be exploited in DNA barcodes for identifying flowering plants this work only highlight the advantages of DNA barcode sequence in primer design and indeed there should be more accurate evaluations of all possible plastids DNA for better design of primers to avoid interferences.

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Antibacterial and Antioxidant Activities of Aqueous Extract of Soapnuts (*Sapindus mukorossi*)

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Abstract

As an alternative to chemical surfactants, the biosurfactants obtained from plants are renewable, biocompatible, biodegradable, less toxic and less expensive. In the present report, the aqueous extract prepared from the pericarps of soapnuts fruits is employed, which are known to exhibit a myriad of biological properties. The extract was characterized using Fourier transform infrared spectroscopy (FTIR) and zeta analyzer for identifying the functional groups and surface charge, respectively. The extract showed abundance of saponins, triterpenoids, flavonoids and negative charge of –8.9 mV. The free radical scavenging and antibacterial activities of the extract were evaluated with DPPH scavenging and well diffusion assays. The DPPH scavenging (%) increased with an increase in extract concentration and showed a significant radical scavenging potential of 85.3% at a concentration of 250 µg/mL. The extract didn't show antibacterial action on Gram–negative bacteria at the selected concentrations. But, it demonstrated significant inhibitory action on Gram–positive bacteria; *Bacillus subtilis* and *Micrococcus luteus* with inhibition zones of 4.0 mm and 12.5 mm at 43.75 mg of crude saponins, respectively. Thus, the green extract used in the present study finds its application as a natural, antibacterial and antioxidant biosurfactant in cosmetic and food industries, as a substitute to chemical surfactants.

Key words: Antibacterial: Antioxidant: Biosurfactant: Saponin: Soapnut

Introduction

The tree *Sapindus mukorossi* (Sapindaceae family) is generally known soapnut, soapberry, washnut, *kunkudu, reetha* etc. The fruits of the tree are extensively used in Asian countries for bathing, washing hair, silk and woolen clothes; kitchen utensils, and polishing tarnished gold and silver ornaments due to its excellent cleansing activity. The pericarps of the fruit are traditionally used in Ayurvedic and folk medicine for curing epilepsy, eczema, psoriasis, migraine etc. The main phytoconstituents of the fruit are saponins (10–11.5%), sugars (10%) and mucilage. The saponins present in the pericarps, main constituent of the aqueous extract are non–ionic glycosides containing sugars such as D–glucose, D–xylose, L–arabinose, L–rhamnose, and glucuronic acid. The saponins are classified under triterpenoidal saponins and mainly of three types i.e. oleanane, dammarane and tirucullane (Fig. 1). The constituent saponin acts as natural surfactant and classified under biosurfactant category. Also, the vitamins present in extract such as A, D, E, and K acts as natural conditioner and impart shine and smoothness to hair, after application. (Suhagia et al., 2011; Upadhyay and Singh, 2012; Yang et al., 2010).

It is significant to note that the extracts of soapnut fruit is known to possess a myriad of

biological properties including hepatoprotective, antiinflammatory, anxiolytic, antiplatelet aggregation, anticancer, antiprotozoal, anti trichomonial, antifungal, antibacterial, free radical scavenging, spermicidal, piscicidal and molluscicidal activities (Ibrahim et al., 2006; Köse and Bayraktar, 2016; Suhagia et al., 2011; Upadhyay and Singh, 2012). As the extract is insecticidal, it is traditionally used for the killing and removal of *Pediculus humanus*, a human lice that infects the scalp (Suhagia et al., 2011; Upadhyay and Singh, 2012). Also, it is known to cure bacterial and fungal based scalp infections, including dandruff by its antidermatophytic activity (Tamura et al., 2001). The saponin containing aqueous extract exhibits functional properties such as superior emulsification activity in comparison with synthetic surfactant sodium dodecyl sulfate. Thus, implying its utilization as a commercial, economical biosurfactant (Ghagi et al., 2011).

Biosurfactants are biological substitutes, derived from plants, bacteria and fungi and typically used as emulsifiers, deemulsifiers, wetting and foaming agents, functional food ingredients and detergents. There are many advantages of plant derived biosurfactants in comparison with the chemical surfactants. They are renewable in nature, sustainable, easily available, less expensive, biocompatible and biodegradable under both aerobic and anaerobic conditions. They also exhibit lower human toxicity and allergenicity; less toxic to environment and helps in remediation of various hydrophobic contaminants in water and soil. Also, their production does not deplete the existing limited petroleum resources (Ghagi et al., 2011; Rao and Paria, 2009; Vijayakumar and Saravanan, 2015).

While, most of the personal care and household products such as soaps, shampoos, body lotions, dishwashing soaps, cosmetics, varnishes, paints, inks etc contain 1, 4–dioxane. It is a byproduct formed during the manufacturing of commercial chemical surfactant/detergent, sodium lauryl sulfate by ethoxylation process. It is volatile, irritant and listed under probable human carcinogen by Environmental Protection Agency (EPA). Notably, the soapnuts are natural and its utilization as a biosurfactant is known for centuries all over the world, widening its application in cosmetic, pharmaceutical, petrochemical, mining, metallurgical, agrochemical and food industries. In this perspective, an attempt has been made to study the antibacterial and free radical scavenging potential of the aqueous soapnut fruit extract. Thus, the study paves a way for finding its application as a natural, antibacterial, antioxidant biosurfactant in our day to day life, an alternate to chemical surfactants.

Materials and Methods

Soapnut fruits were obtained from the local market. Absolute ethanol (Shymlakhs International, London, UK), 1, 1 diphenyl picryl hydrazyle (DPPH) (Thomas Baker Chemicals Pvt. Ltd, Mumbai, India), streptomycin sulphate (Sigma–Aldrich, Bengaluru, India), nutrient broth and Mueller Hinton agar (HiMedia Chemicals Pvt. Ltd, Mumbai, India) were used during this study. The medium nutrient broth made up of sodium chloride (5 g/L), yeast extract (1.5 g/L), peptone (5 g/L) and beef extract (1.5 g/L). The Mueller Hinton agar (pH 7.4 ± 0.2) composed of starch (1.5 g/L), beef extract (2 g/L), casein acid hydrolysate (17.5 g/L) and agar (20 g/L). All the solutions were prepared in ultra pure water of 18.2 MΩ–Cm resistivity; produced from Elga Purelab Flex 3 water polishing unit (High Wycombe, England). At 121ºC for 20 min, glassware, plasticware and media used in the present study were sterilized in Obromax vertical autoclave (Delhi, India).

Preparation of aqueous extract of soapnut pericarps: The soapnut fruits were dried at 50ºC in Osworld JRIC–7/A laboratory hot air oven (Mumbai, India) for 24 h. The acquired pericarps after removal of the seeds were pulverized into fine powder using Prestige Deluxe–Vs mixer grinder (Bengaluru, India) and sieved (Jayant Scientific Industries, Mumbai, India) to obtain a

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particle size of 300 µm. The collected powder was stored in an air tight container at room temperature. A 15% (w/v) aqueous solution of the pericarps in ultrapure water was prepared by continuous stirring with Tarson Spinit magnetic stirrer (Kolkata, India) at room temperature for 2.5 h. The extract was centrifuged using Remi R–24 Research centrifuge (Mumbai, India) at 8, 000 rpm for 10 min and the obtained supernatant was filter sterilized with sterile Millipore 0.22 µm syringe filter (Bengaluru, India). Thus, the obtained aqueous extract was used for all the studies (Fig. 2).

Characterization of the aqueous soapnut extract: The zeta potential of the extract was determined with Malvern Zetasizer Nano ZS90 (Malvern, UK). Using Labconco Freezone 4.5L Plus benchtop cascade freezedry system (Kansas City, USA), the aqueous extract solution was made into powder. The IR spectrum of the lyophilized powder was recorded at a wave number range of 1000–4000 cm^{-1} with Bruker Optics TENSOR 27 FTIR spectrometer (Ettlingen, Germany),

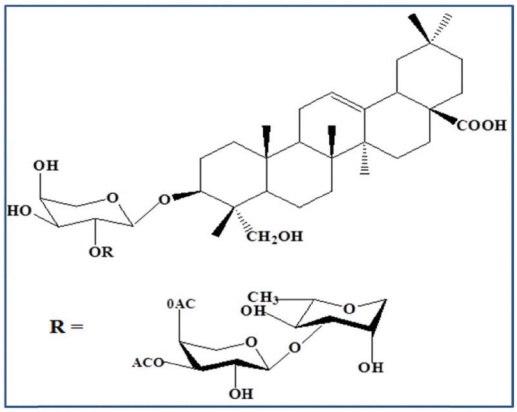

Fig. 1. The typical structure of a saponin from *Sapindus mukorossi* (Rao and Paria, 2009)

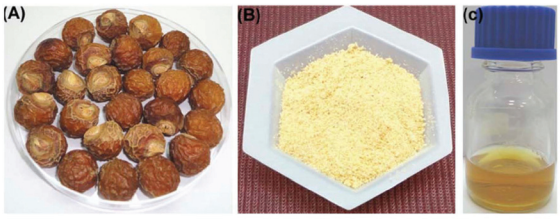

Fig. 2. Digital photographs showing the soapnut (a) dried fruits, (b) powder and (c) aqueous extract (15%)

DPPH scavenging activity: The antioxidant activity of the extract was investigated by scavenging (1'–1' diphenyl picryl–hydrazyle) (DPPH). The DPPH is a purple coloured, stable free radical. The solutions of 100 µM DPPH prepared in absolute ethanol were mixed with 62.5–100 µg/mL concentration of the extract and incubated in dark at room temperature for 60 min. The ascorbic acid solution (50 µg/mL) and water were used as positive and negative controls, respectively. The absorbance was noted at 520 nm with Analytic Jena AG Specord 200 Plus UV–visible spectrophotometer (Jena, Germany). The DPPH scavenging (%) by applying the equation: DPPH scavenging (%) = (DPPH absorbance–sample absorbance/DPPH absorbance) × 100 (Kora and Rastogi, 2018). The experiment was carried out in triplicate and the values were expressed as mean ± SD.

Antibacterial activity: The antibacterial activity of the extract was checked with well diffusion method. American Type Culture Collection (ATCC) strains, *Escherichia coli* 25922 and *Pseudomonas aeruginosa* 27853; and *Bacillus subtilis* 6633 and *Micrococcus luteus* 10240 were used as representive test strains for Gram–negative and Gram–positive bacteria, respectively. The Mueller Hinton agar plates were inoculated with bacterial suspension by spread plate method. The suspension was prepared from the nutrient broth grown overnight culture by turbidity adjustment to 0.5 McFarland standard. The aqueous extract containing 12.5, 25, 37.5 and 43.75 mg of crude biosurfactant were added to the 8 mm diameter wells made in the solid agar medium. The negative and positive control wells were maintained with water and the antibiotic streptomycin (10 µg), respectively. The plates were incubated at 37ºC

for 24 h in Remi CIS–24 Plus bacteriological incubator (Mumbai, India). The inhibition zone was calculated by deducing the well diameter from the total inhibition zone diameter. The average value was obtained from the three independent experiments carried out with each bacterial strain (Kora and Rastogi, 2018).

Results and discussion

Fourier transform infrared spectroscopy (FTIR): The IR spectrum of the lyophilized aqueous extract was noted for identifying the functional groups of the biomolecules present in the soapnut pericarps (Fig. 3). The major absorbance bands in the spectrum are at 3343, 2924, 2855, 2130,

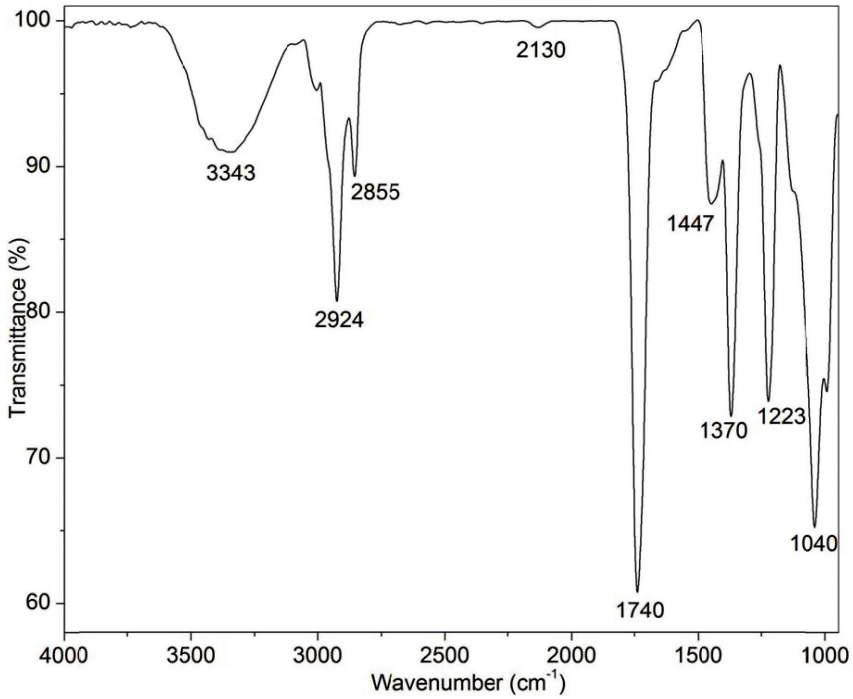

Fig. 3 The FTIR spectrum of freeze dried powder of the soapnut aqueous extract

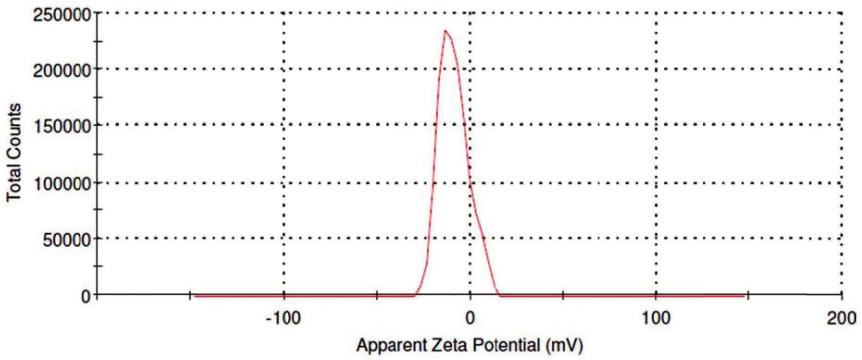

Fig. 4 The zeta potential distribution of the soapnut aqueous extract

1740, 1447, 1370, 1223 and 1040 cm^{-1}, respectively. The broad band observed at 3343 cm^{-1} could be assigned to stretching vibrations of hydroxyl groups in alcohols and phenolics. The bands at 2924 and 2855 cm^{-1} correspond to respective asymmetric and symmetric stretching vibrations of methylene groups. The broad band at 2130 cm^{-1} arises from various carbonyl species. The peak at 1740 cm^{-1} could be assigned to carbonyl stretching vibrations in aldehydes, ketones and carboxylic acids. The symmetrical stretch of carboxylate group can be attributed to the bands present at 1447 and 1370 cm^{-1}. The peaks at 1223 and 1040 cm^{-1} correspond to C–O stretch of phenolic and alcoholic groups, respectively. Hence, the distinctive peaks observed in the spectrum denote the abundance of various bioactive molecules such as saponins, triterpenoids, flavonoids, fatty acids etc in the fruit extract (Du et al., 2014; Sharma et al., 2013; Suhagia et al., 2011). The aqueous extract exhibited a zeta potential value of –8.9 mV (Fig. 4). The negative charge of the extract is due to the presence of various saponins in the extract (Rao and Paria, 2009).

Antioxidant activity*:* The antioxidant capacity of the extract was quantified with DPPH scavenging assay (Fig. 5). With time, the colour of the DPPH solution containing the soapnut extract slowly changed from purple to pale yellow (Inset of Fig. 5). The DPPH scavenging (%) increased from

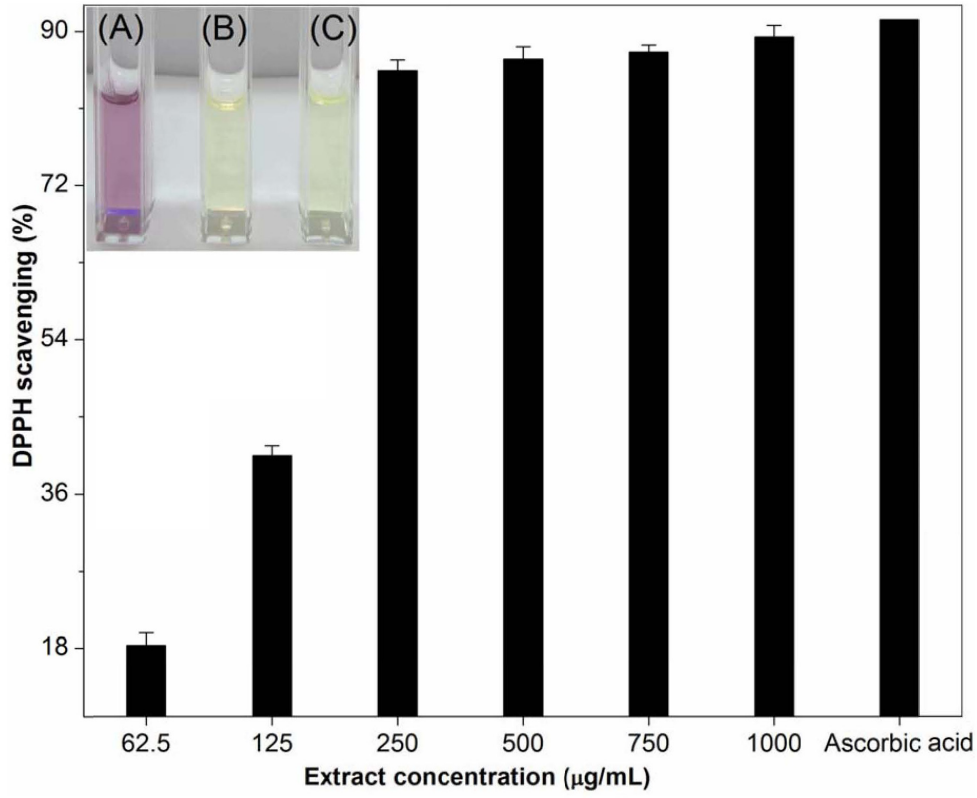

Fig. 5 The DPPH radical scavenging activity of soapnut aqueous extract at different concentrations (62.5–1000 µg/mL). Inset: DPPH solution colour (a) before and after treatment with (b) soapnut extract (250 µg/mL) and (c) ascorbic acid (50 µg/mL)

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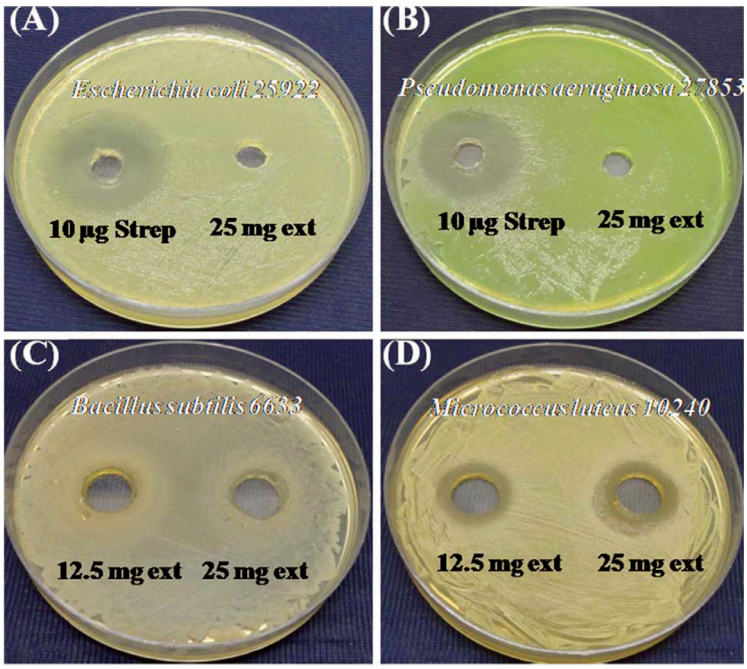

Fig. 6. Bacterial culture plates showing the inhibition zones around the wells loaded with different amounts of soapnut extract and streptomycin, (a) *Escherichia coli* 25922, (b) *Pseudomonas aeruginosa* 27853, (c) *Bacillus subtilis* 6633 and (d) *Micrococcus luteus* 10240

18.1–85.3% with an increase in extract concentration from 62.5-250 µg/mL. Further, the value was marginally increased and reached 89.3% at 1000 µg/mL. Whereas, the positive control ascorbic acid at 50 µg/mL concentration showed a scavenging activity 91.3%. The negative control water did not show any colour change from purple. The extract demonstrated significant radical scavenging potential at a concentration of 250 µg/mL. Thus, the extract is meeting the requirements of an effective antioxidant. The exhibited antioxidant activity of the extract could be attributed to the reducing and chelating properties of the different complex biomolecules of the extract, including saponins, phenolics and flavonoids (Bahri-Sahloul et al., 2014; Kora and Jayaraman, 2012).

Antibacterial activity*:* The well diffusion assay was used for evaluating the antibacterial activity of the extract. The results showed that there was no antibacterial action on Gram–negative bacteria at the selected concentrations of the extract. While, the extract demonstrated significant inhibitory action on Gram–positive bacteria (Table 1). For *B. subtilis*, the inhibition zones ranged from 2.5-4.0 mm in the range of 12.5–43.75 mg of crude saponins. In the case of *M. luteus*, the inhibition zones were higher and ranged from 8.0–12.5 mm. Interestingly, the highly inhibited *M. luteus* strain is a part of the normal flora of the mammalian skin and commonly colonizes on mucosal tracts of humans. As expected, the positive control streptomycin showed higher inhibition action on all the test strains at 10 µg, ranged from 24.6–30.0 mm.

These results are in accordance with earlier study carried out with polyphenolic rich callus culture extracts of *Crataegus azarolus* L. var.

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Table 1. The inhibition zones observed with different bacterial culture plates loaded with soapnut extract and streptomycin

Test compound	Zone of inhibition (mm)*			
	***E. coli* 25922**	***P. aeruginosa* 27853**	***B. subtilis* 6633**	***M. luteus* 10240**
Extract (12.5 mg)	0	0	2.5 ± 0.7	8.0 ± 0
Extract (25 mg)	0	0	3.5 ± 0.7	9.0 ± 0
Extract (37.5 mg)	0	0	3.8 ± 0.5	10.0 ± 0
Extract (43.75 mg)	0	0	4.0 ± 0	12.5 ± 0.7
Streptomycin (10 µg)	29 ± 0	24.6 ± 1.5	28.3 ± 0.5	30.0 ± 0

*Values are mean ± SD (*n* = 3).

aronia. The differential activity of the extract towards Gram–positive and Gram–negative bacteria is based on the difference in the cell wall structure. The hydrophilic outer membrane of the Gram–negative bacteria acts as a permeability barrier, thus exhibits resistance towards antibacterial compounds. Whereas, the lipophilic cell wall of Gram–positive bacteria facilitates the penetration of hydrophobic compounds. The inhibition of Gram–positive bacteria by aromatic compounds could be due to the inhibition of enzyme production, cell wall deterioration and cell lysis (Bahri-Sahloul et al., 2014). Previous study on antimicrobial activities of pericarp saponins showed moderate growth inhibitory action only on Gram–positive bacteria, but not against Gram–negative bacteria (Tamura et al., 2001). The data is further supported from the earlier reported study on preservative efficacy of crude saponin extract. It is reported that the extract is an effective preservative against Gram–positive *Staphylococcus aureus*, but ineffective against Gram negative *E. coli* (Yang et al., 2010).

The current study employs the aqueous extract obtained from the pericarps of soapnuts as an antioxidant and antibacterial agent. The saponins present in the extract act as renewable, biocompatible, biodegradable and non–toxic biosurfactant. The IR analysis reveals the abundance of various bioactive molecules such as saponins, triterpenoids, flavonoids, fatty acids etc in the extract. The extract exhibited significant radical scavenging potential at 250 µg/mL concentration, thus qualifies as an effective antioxidant. Also, the extract showed inhibitory action on Gram–positive bacteria. The results of the present report highlights the applications of the soapnut extract in various fields including cosmetics and food. It has potential as a functional food additive/supplement during chemotherapy and antibiotic treatment. It could be utilized as a substitute for the chemical surfactants in conditioners and hand wash for effective control of skin bacterial flora, with no side effects.

Ethics Statements

Not applicable.

Conflict of interest

The author declares no conflict of interest.

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Computational and Experimental Validation of Methotrexate as *Staphylococcal* - DHFR inhibitor

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Abstract

Dihydrofolate reductase (DHFR) is an enzyme that reduces dihydrofolate to tetrahydrofolate for the *denovo* synthesis of purines. DHFR is a well-established and classic drug target used in cancer therapy. Several drugs like Methotrexate (MTX), Trimethoprim, Pemetrexed, Pyrimethamine, etc. are known inhibitors of DHFR and presently used to treat cancer patients. Staphylococcus is a major human pathogen and the causative agent of numerous hospital and community-acquired infections. In the present investigation, DHFR of *Staphylococcus aureus* (PDB Id: 2W9G) was subjected to molecular docking to evaluate whether the anti-cancer drugs, MTX and Pemetrexed strongly bind to the former. The results of molecular docking indicated that MTX and Pemetrexed strongly interact with Staphylococcal DHFR with the binding energy of -8.3kcal/mol and -9.0kcal/mol respectively. To validate the *in silico*studies, the antimicrobial property of MTX and pemetrexed was evaluated in clinical strains of *S. arlettae* and *S.sciuriin vitro* and the results indicated that MTX but not pemetrexed possessed antimicrobial activity. But the similar antimicrobial effect of the above-mentioned drugs was not found in gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27853), and *E. coli* (ATCC 25922). The Minimal Inhibitory Concentration (MIC) of MTX was found to be higher than 2mg/ml for both strains. Even though the MIC values of MTX are high, we propose that structural modification of MTX or its combination with conventional antibiotics may lead to the discovery of the new potential antimicrobial drug.

Key words: DHFR, molecular docking, anti-cancer drugs, drug repurposing, antimicrobial drug.

Introduction

Staphylococcus is a gram-positive, spherical facultative anaerobe that belongs to the family Staphylococcaceae. They usually inhabit the nasopharynx, skin and urogenital tract of humans (1). The infections caused by these bacteria are generally categorized under 'staph infections', which are mostly hospital-acquired. In the modern antibiotic era, where thousands of diverse antimicrobial drugs have been produced, the increasing antibiotic resistance of pathogens have escalated the need for the discovery of new potent drugs. The potential of dihydrofolate reductase (DHFR) as a therapeutic target for treating infections has been substantiated a century back (2). DHFR reduces dihydrofolate to tetra-hydrofolate using NADPH as a co-factor and plays an important role in the synthesis of purines.Folic acid (pteroylglutamic acid) is a natural substrate of DHFR and is composed of three components: pteridine ring, *para*-amino benzoic acid and glutamic acid. MTX, a folate analogue differs from

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folic acid by substitution of amine for hydroxyl group at N4 position of pteridine ring. Due to structural similarity of MTX with folic acid, the former strongly interacts with DHFR and therefore interfere with nucleic acid synthesis and used as an anti-cancer agent (3). Pemetrexed, an antimetabolite anticancer agent contains pyrrolopyrimidine ring in place of pteridine ring, and apart from DHFR it also inhibits two additional enzyme, thymidylate synthase, and glycinamide ribonucleotide formyltransferase (4).

Drug repurposing or repositioning refers to the process of discovering new uses of existing drugs. An example of drug repurposing is the approval of antifungal agent amphotericin B for the treatment of visceral leishmaniasis (5) and the repurposing of the anti-malarial drugs chloroquine for effective treatment of amoebiasis and toxoplasmosis, respectively (6). Even though there are several reports of drug repurposing against bacterial pathogens, none has been approved by the FDA till date. Moreover, drug repurposing offers several advantages compared

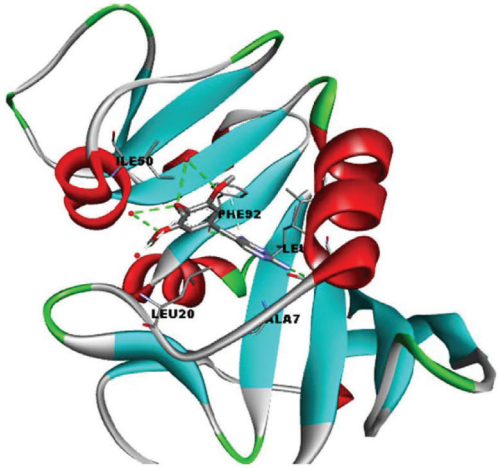

Fig. 1: Active site *Staphylococcus aureus*-DHFR with Trimethoprim. The amino acids in the active site involved in interaction with trimethoprim are shown

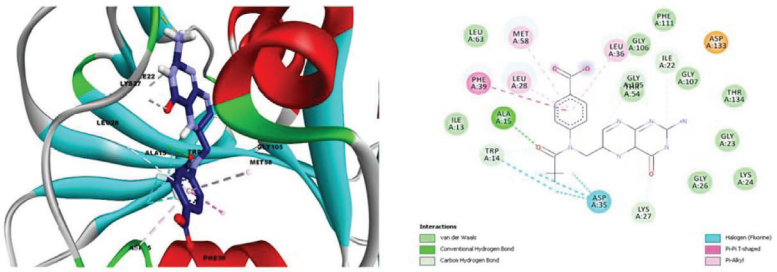

Fig. 2: Staphylococcal DHFR-MTX interaction. **A**) 3D representation of interaction between MTX and residues in DHFR ligand binding pocket. **B**) 2D interaction diagram of MTX with residues in DHFR ligand binding pocket.

to *de novo* drug development for antimicrobial drug discovery like accelerated drug development process, reduced cost, bypassing the majority of the discovery and preclinical stages, etc. (7).

Molecular docking is a powerful approach for structure-based drug discovery and is being increasingly used for the drug discovery process.In this context, the present investigation was carried out to understand the binding mechanism of two anticancer antifolate drugs, viz MTX and pemetrexed with DHFR of *Staphylococcus* by using molecular docking approach followed by the experimental validation of antimicrobial activity.

Materials and methods

Selection of protein (DHFR) for docking: The crystal structure of isolated DHFR from a Wild type *Staphylococcus aureus* withPDB ID: 2W9G was retrieved from protein database (https://www.rcsb.org/structure/2W9G).The structure ofMTX and pemetrexed were retrieved from PubChem, an open chemistry database atNational Institute of Health (NIH). The ligand binding sitescomprising residues Asp271, Leu20, Phe92, Thr46 and Leu5 were identified in the crystal structure (Fig 1).

Molecular docking: Molecular docking is the computer simulation of the binding affinity of ligands with the receptor.The molecular docking was performed by using AutoDock Vina (version-1.5.6) with Chimera(version-1.13.1) used as an interface. The energy minimization was performed bothfor ligand and receptor and subsequently, mol2 files were generated. The grids size and position were calculated in such a way that all the amino acid residues in the active sites of DHFR were accommodated. The centre grid coordinates used inthis study for AutoDock Vina were 12×17×6 while size grids used for x, y, z were28×31×34 respectively. The number of binding modes was fixed at 5. The rest of theparameters were kept in default.Discovery Studio 4.1 visualizer (http: //accelrys.com/products /discovery-studio/) was used for the visualization of docking results

Collection of clinical isolates of Staphylococcus: Two clinical strains of *Staphylococcus* (GC03 and GC04) were obtained from the State Public Health and Clinical Laboratory, Trivandrum and maintained in nutrient agar. The species identification of the sample was done by using VITEK 2 VERSION: 07.01 from CEPCI Laboratory and Research Institute, Kollam.

Antimicrobial assay: The antimicrobial assay was done in Gram-positive bacteria-*Staphylococcus* (GC03 and GC04) and in Gram negative bacteria- *Pseudomonas aeruginosa* (ATCC27853) and *Escherichia coli* (ATCC25922). 15-20 ml of Nutrient agar was poured on glass Petri plates and allowed to solidify. Holes of 7 mm width were made in agar usinga sterile well borer. Afterward, 500 µl of inoculum (0.5 McFarland standard) was poured on thesurface of the solidified agar and spread by plate spreader. The excess inoculum was removedand plates were kept in biosafety hood for 10 minutes. Then the wells were filled with0.5mg and 1.0 mg of MTX (sigma) and Pemetrexed (sigma) respectively and incubated at 37°C for 16 hrs.

Detection of minimum inhibitory concentration: The overnight culture of GC-03 and GC-04 was diluted to achieve a turbidity equivalent to a 0.5 McFarland standard. One mL of the adjusted inoculum was added to each tube containing 1 mL of MTX or pemetrexed each in the dilution series (0.5mg/ml, 1mg/ml, 1.5 mg/ml and 2 mg/ml). The tubes were Incubated at 37 ºC for 16 hrs. The lowest concentration at which the isolate is completely inhibited (as evidenced by the absence of visible bacterial growth) was recorded as the minimal inhibitory concentration or MIC.

Results

The affinity of MTX and pemetrexed with DHFR of *Staphylococcus aureus* was examined by molecular docking approach. The list of the compounds docked, canonical smiles, molecular weight, docking score and intra-molecular Hydrogen bonds formed are shown in Table 1. The Auto Dock binding energy of MTX and

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pemetrexed were found to be - 8.3 and -8.7 kcal/mol respectively. The ligand poses with the highest binding energy and RMSD=0 was selected as the best pose. Thehydrogen bonds play the most significant role in the interaction between receptor and ligand in molecular docking (8). MTX forms hydrogen bonds with ALA15 of the binding pocket in DHFR whereas Pemetrexed forms hydrogen bonds with ALA15 and ALA 53 of the binding pocket in DHFR (Figures 2 and 3). Other than hydrogen bond, MTX is also involved in Pi-Alkyl, Pi-sulphur and Pi-Pi Stacked interactions whereas Pemetrexedis involved in Pi-Alkyl, Carbon-Hydrogen bond, Pi-sulphur, Pi-Pi Stacked interactions with DHFR (Table 2).

MTXblocks the action of human dihydrofolate reductase (DHFR), thereby inhibiting the metabolism of folic acid (9). Based on the docking scores, it was hypothesized that the MTX and pemetrexed also inhibit DHFR of *Staphylococcus* by strongly binding to it. To validate this, we further experimentally evaluated the antimicrobial activity of MTX and Pemetrexed against Staphylococcal-DHFR. The *Staphylococcus* (GC03 and GC04) were identified using VITEK 2 system as *Staphylococcus arlettae* and *Staphylococcus sciuri* respectively.The size of the zone of inhibition for MTX at concentrations, 0.5 mg and 1.0 mg was measured as 14mm and 16mm in GC03 and 14mm and 17mm in GC04 respectively (Figure 3). However, with Pemetrexed no zone of

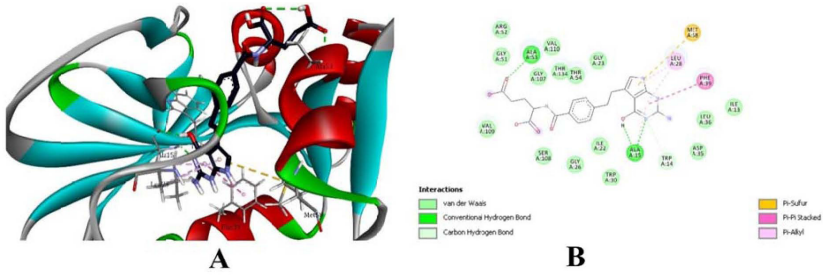

Fig. 3: Staphylococcal DHFR-Pemetrexed interaction. **A**) 3D representation of interaction between Pemetrexed and residues in DHFR ligand binding pocket. **B**) 2D interaction diagram of Pemetrexed with residues in DHFR ligand binding pocket.

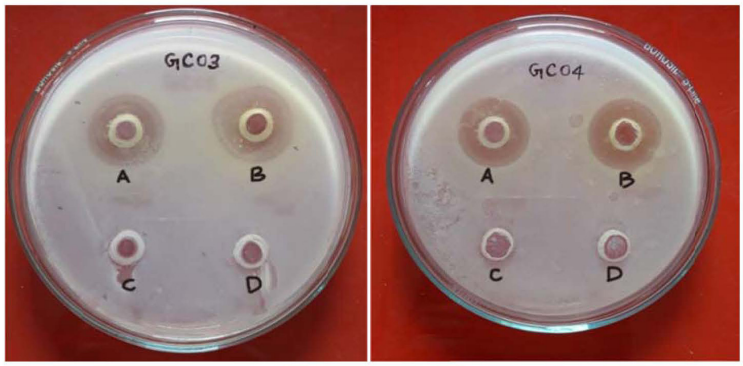

Fig. 4: Antimicrobial assay of MTX and Pemetrexed against *Staphylococcus* (GC03 and GC04). (A) 0.5 mg of MTX(B) 1.0 mg of Pemetrexed (C) 0.5mg of Pemetrexed (D) 1.0 mg of Pemetrexed.

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Table 1: Table showing list of compounds docked, canonical smiles, molecular weight, docking score and number of intra-molecular hydrogen bonds formed between ligand and receptor.

Sl. No	Compounds	Canonical Smiles	Mol. Weight (g/mol)	Autodock vina binding energy (kcal/mol)	No. of Intramolecular H-bond formed
1	Methotrexate	CN(CC1=CN=C2C(=N1)C(=NC (=N2)N)N) C3=CC=C(C=C3)C (=O)NC(CCC (=O)O)C(=O)O	454.4	-8.3	1
2	Pemetrexed	C1=CC(=CC=C1CCC2= CNC3=C2C(=O)NC(=N3)N)C (=O)NC(CCC(=O)O)C(=O)O	427.417	-8.9	2

Table 2: Table showing various types of interaction between MTX and pemetrexed with DHFR.

Sl. No.	Compounds	Residues of active site involved in H-bonding	Other Interaction
1	Methotrexate	ALA15	Pi-Alkyl, Pi-Pi T Shaped, Halogen (Fluorine), Carbon-Hydrogen bond
2	Pemetrexed	ALA15, ALA53	Pi-Alkyl, Carbon-Hydrogen bond, Pi-sulphur, Pi-Pi Stacked

inhibition was noticed which revealed that onlyMTX possessed antimicrobial activity. (Figure3). In *P. aeruginosa* and *E. coli,* no zoneswere observed for Pemetrexed and MTX (data not shown). The results of antimicrobial assay revealed that MTXdisplayed antimicrobial activity in both GC03 and GC04while Pemetrexed does not have any antimicrobial activity at given concentrations. We inferfrom this experiment that MTX inhibits the growth of Gram-Positive bacteria and notGram-Negative bacteria. However, Pemetrexed has no activity either in Gram-Positive orGram-Negative bacteria. To the best of our knowledge,this is the first report of the antimicrobial activity of MTX against *S. arlettae* and *S.sciuri*. Overall, the antimicrobial activities exhibited by MTX validate the resultsobtained by molecular docking. The results of MIC testing revealed that the growth of GC-03 and GC-04 was not inhibited up to the concentration of 2 mg/ml, suggesting MICe"2.

Discussion

In the present investigation, an attempt was made to evaluate whether the antifolate, anti-cancerdrugs possess the ability to bind Staphylococcal DHFR, by molecular docking followed by theexperimental validation. With the help of molecular docking approach, we predicted that MTX andPemetrexed might inhibit Staphylococcal DHFR. The results of antimicrobial assay demonstrated thatMTX possesses antimicrobial activity but not Pemetrexed, even though the latter showedhigh docking score (-8.9 kcal/mol). Molecular docking has become an increasingly importanttool for small molecule drug discovery (10). The molecular dockingapproaches have led to the identification of several ligands/ drug-like molecules as well astarget receptors in the past (11,12,13). Themajor limitation of molecular docking is the lack of confidence in the ability of scoringfunctions to give accurate binding energies. This is due to the fact that some

intermolecularinteractions, such as solvation effect, entropy changes,(14) halogen effect onprotein-ligand affinity (15) and the water molecule forming a bridge between ligand and receptor,(16) cannot be predicted accurately by molecular docking.The Aforementioned reasons might be responsible for the inability of Pemetrexed to inhibit thegrowth of *Staphylococcus in vitro*. MTX is an established antimetabolite that blocks the action of dihydrofolatereductase, thereby hindering the metabolism of folic acid. It has been used widely since the1950s to treat a variety of neoplastic and inflammatory diseases (9). Theantimicrobial studies and MIC value suggest that MTX has weak antimicrobialactivity and could not be used as a choice of drug to treat staphylococcal infections. Previously, Kruszewska et al., 2000 (17) have shown the antimicrobialactivity of MTX against *Staphylococcus aureus* strains with high MIC values.Our results are in strongagreement with the previous studies showing antimicrobialactivity and high MIC values of MTX against *S.aureus*. Howeverrecent studies on drug repurposing have emerged as an alternative approach to rapidly identifyeffective drugs and drug combinations to combat drug-resistant bacteria.Soo et al., (2016) have identified two potent anticancer medications 5- fluorouraciland 6-thioguanine possessing antibacterial property (18). The authors further hypothesized thatanticancer drug combination with conventional antibiotics (Synergism) have the potential forclinical trials to treat multidrug-resistant *Acinetobacter baumannii* infections. A recent studyon the combination of the anticancer agent mitomycin C and Tobramycin-Ciprofloxacin further enhanced the already potent antibacterial activity of mitomycin in multiple drug resistant clinical isolatesof Gram-negative bacteria (19). Similar synergistic activity ofMTX with conventional antibiotics is yet to be examined and might be a promisingstrategy to combat bacterial infection. It is not clear whether MTX would be a safe antimicrobial agent. However, there is a need for new analogues of MTX and novel drug combinations of MTX with conventional antibiotics have to be developed to treat lethal multidrug-resistant bacterial infection. In thisrespect, MTX alone or in combination may represent alternative to existing antibioticsfor the treatment of bacterial diseases. Moreover,the anti-biofilm effect of MTX has not been reported and is another challenging areawhich needs attention.

Conclusion

In the present investigation, DHFR of *S. aureus* (PDB Id: 2W9G) wassubjected to molecular docking to identify a novel antimicrobial agent. Molecular docking studies predicted that MTX and Pemetrexedstrongly bind to DHFR of *S. aureus*. The results of antimicrobial assay revealed that MTX but not Pemetrexed inhibit *Staphylococcus*. MTX and Pemetrexed did not inhibit the growth of gram-negative bacteria i.e, *P. aeruginosa* and *E. coli*. The Minimal Inhibitory Concentration (MIC) of MTX was found to be morethan 2mg/ml for both strains. Even though the MIC values of MTX are high, we propose that structural modification of MTX or its combination with conventional antibiotics may lead to the discovery of new potential antimicrobial drugs.

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Unlocking the Therapeutic Potential of *Syzygium cumini* Seeds Extract

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Abstract

This work provided initial evidence that the methanol and ethanol extract of *Syzygiumcumini* seeds polyphenol compounds are separated. The major polyphenolic compounds are determined and its derivatives posses strong amylase and tryosinase inhibitory activities with IC_{50} values more than 50µg/ml for amylase and more than 80µg/ml for tryosinase respectively. Such half maximal inhibitory concentration (IC50) values substantiate to manage diabetes and neuroprotective effects in patients to minimize the secondary complications associated with diabetes and melanogenesis.

Key words: *Syzygium cumini*; tyrosinase; amylase activity; anti-diabetic

Introduction

In the plant kingdom phenolic compounds are widely distributed in the plants many studies have been conducted in order to evaluate the biological activities of extracts of several usual and unusual sources of polyphenols. There is remedy for every disease (1). Plants were the main source of drugs for the world's population still relies on those plants and other tools of traditional medicine (2). Plants have provided a source of inspiration for novel drug compounds, as plant-derived medicines have made large contributions to human health and wellbeing. Medicinal plants are the richest bioresource of drugs for traditional system of medicine, neutraceuticals, food supplements, modern medicine, pharmacy intermediates, folk medicines and chemical entities for synthetic drugs (3,4). Since the use of medicinal plants based drugs contain least or no side effects they are considered great importance to the health of individuals and communities (5,6).

The medicinal plants are useful for healing as well as for curing human diseases because of presence of various phytochemical constituents (7). Phytochemicals like phenolic compounds are naturally occurring in the medicinal plants, have defence mechanism, and protect from various diseases (8, 9). Medicinal plants parts are commonly rich in phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins.

Syzygium cumini is distributed throughout Tropical Asian and Indian countries. It is important indigenous plant of the family Myrtaceae. It is produces fruits in various sizes, which is under utilized (10). The fruit pulp is sweet and seeds are acrid, sour and tonic. They have biological activities such as hypoglycemic, hypolipidemic, cardio protective, hepatoprotective, antibacterial, anti-inflammatory and anti-allergic effects have been reported previously with consumption of *S. cumini* (11,12). It is rich in polyphenols and contains compounds such as flavonoids, anthocyanin,

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tannins, terpenes and aliphatic acids (13,14). There are large amount of phytonutrients, which have been reported for their anti-allergic, anti-inflammatory, anti-diabetic, anticancer and antioxidant properties studies revealed that *S.cumini* seeds have great nutritional and pharmacological potentials.

S. cumini it is also used as antioxidant, anti-inflammatory,neuropsycho-pharmacological, antimicrobial,anti-bacterial, anti-HIV, anti-malarial andantifungal, nitric oxide scavenging, antidiarrheal, antifertility, anorexigenic, gastro protectiveantioxidant activity, anti-ulcerogenicand radio protective activities (15).

The seed powder of *S. cumini* is used by the diabetic patients to control the blood sugar level naturally, pulp of this seeds is used to treat diabetes, digestion problems and as diuretic.

In the present study, the preliminary phytochemical screening of the subject plants reveals that they are good sources of natural products. The results concluded that the studied plants of *S. cumini* contained appreciable amount of tannins, flavonoids, steroids, alkaloids, and saponins however terpenoidscontent was almost negligible. They are also served to be the potential source as antidiabetic and neuro protective disorder (16).

Antioxidants are molecules whose main function is to scavenge the free radicals. They can scavenge the free radicals even before they generated. Antioxidants actually tries to upgrade defense mechanism of the body the most common natural source of antioxidants are plants, mainly present in fruits and vegetables in the form of polyphenolic compounds, which are mainly flavonoids. It is particularly important for those people who do not consume enough fruits and vegetables in their daily diet (17, 18).

Materials and Methods

Apparatus and Materials: Materials are 100ml of measuring cylinder, 5ml of measuring cylinder, 50ml beaker and cylinder, test tubes, reagent bottles, Thin layer chromatography sheets, column chromatography, Soxhlet apparatus, ruler.

Chemicals and reagent: Distilled water, methanol, ethanol, petroleum ether, chloroform, Dimethyl sulfoxide, glacial acetic acid, ferric chloride, sodium hydroxide, silica powder mesh size (100-200), dilute HCL, 5% ferric chloride, 10% ferric chloride, Meyer's reagent, Folin'sChiocalteu reagent, DPPH (2,2-diphenyl-1-picrylhydrazyl), Enzyme alpha amylase and tyrosinase from sigma aldrich, India. And other solvents from high purity grade.

Plant materials and Collection: *S.cumini* seeds was purchased from local super market, Chennai, India. The seeds ware collected and washed properly with water. They are dried under sun shade for 24 hour. The dried seeds are made to fine powder fine powder by using an electrical grinder and the powder was stored in airtight container to avoid the effect of humidity and moisture (19).

Preparation of Extract: The powder form of plant seeds was weighed for 10g each. The solvents were selected based on their solubility. Methanol and Ethanol were selected for *S.cumini* seeds as they soluble most of the compounds. Extracts were prepared by using Soxhlet apparatus. It consists of condenser, thimble, boiling flask and mantle. It is set at 65 ºC and is vaporized. 10g powder is weighed and packed to the thimble by placing cotton before loading sample, 100ml of solvent is poured to the heating flask. They are continued for 5 cycles for 4hrs. The extract were collected for about 50ml and are evaporated using Rotary evaporator at 40ºC. The paste form of sample is suspended with solvent and kept in a glass container and stored at -18ºC for further biochemical assay (20).

Phytochemical screening: The crude ethanol extracts of S.*cumini* seeds was tested for the presence of bioactive compounds by using following standard methods.

Test for Alkaloids: To the 2ml of extract add few few drops of Meyer's reagent. Formation of white precipitate indicates the presence of alkaloids.

Test for phenol: To 2ml of extract, 5% $FeCl_2$ solution was added. Deep blue-blackcolour indicates presence of phenol.

Test for flavonoids: To the test solution, add few drops of $FeCl_2$solution, intense green colour was formed to show the presence of flavonoids.

Test for tannins: Some amount of extract was dissolved in distilled water to this solution 2ml of 5% $FeCl_2$solution was added. Formation of blue green indicates presence of tannins.

Test for glycosides: To 2ml of test solution, 3ml of glacial acetic acid and 1 drop of 5% $FeCl_2$were added in a test tube. Add carefully 0.5ml of concentrated sulphuric acid by the side of the test tube. Formation of blue colour in acetic acid layer indicates the presence of cardiac glycosides.

Test for saponins: To 5ml of extract, few drops of sodium bicarbonate is added and shaken well and kept undisturbed for 2 minutes. A honey comb like froth was formed indicates presence of saponins.

Test for terpenoids: To 1ml of extract and 2ml of chloroform and 5 to 10 drops of concentrated sulfuric acid. Formation of reddish brown colour indicates presence of terpenoids.

Total phenol estimation: Total phenol content in both seed extract were assessed based on the reduction of phosphotungstic acid to phosphotungstic blue and as result absorbance increases due to rise in the number aromatic phenolic group. For the purpose 100µl of each prepared extract was separately added to the test tubes each containing 250µl of folin's reagent and 750µl of 20% sodium carbonate solution and final volume is made up to 5ml with distilled water. After 30 minutes incubation at a dark room, absorbance is taken at 765 nm using of UV visible spectro photometer against control having distilled water except sample (21).

Total polyphenol was estimated and values are expressed as Gallic acid equivalent (mg GAE/ 100g).

C= c ×V /m Where,

C =TPC (mg/100g plant extract in GAE)

c= concentration of Gallic acid (mg/ml)

V= volume of extract (ml)

M= weight of seed (g)

Antioxidant Assay

DPPH scavenging assay: The Antioxidant activity of ethanol and methanol extract of *S.cumini*was measured on the basis of the scavenging activity of stable 1,1-diphenyl 2-picrylhydrazyl (DPPH) characterized as a free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalisation also gives rise to the deep violet colour, characterized by an absorption band in ethanol solution centered at about 517nm. When the solution DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour to pale yellow colour. Chemicals used are 1,1-diphenyl 2-picrylhydrazyl (DPPH), methanol, Ascorbic acid standard concentration of 1mg/ml in methanol. And sample extract concentration from 100mg/1ml solvent (22).

1ml of 0.1mM DPPH solution in methanol was mixed with of various concentration (20µl to 100µl) of plant extracts. The mixture was then allowed to incubate for 30 minutes in dark room. Distilled water was used as the blank measurements in the experiment. After 30 minutes of incubation the reduction of the DPPH free radical was measured by reading the absorbance at 517nm. Ascorbic acid was used as standard. The percentage of 50% inhibition IC_{50} was found.

Radical Scavenging activity is measured by the following formula,

RSC (%) = ((Abs control – Abs sample)/Abs control)) × 100.

TLC profile : Thin layer chromatographic plate (5 × 20 cm) 0.5mm thickness was used. The sample

of methanol extract of *S.cumini*was spotted manually using a capillary tube. The plate was developed in Butanol : Methanol with equal ratios as solvent system. After development they were viewed under uvtransilluminator to visualize spots.

Column Chromatography: Fractionation with column chromatography, columns of silica gel (100 to 200 mesh size) was washed with 20ml of respective solvents that is methanol for *S. cumini* was mixed to silica and made into a slurry and poured to the column and packed. A 10ml of each sample extract is loaded to the column slowly by the sides of the column by closing the stopcock and after the level of solvent is reached open the stopcock and collect the elutes at various time period and stored in cold temperature. 10 elutes from each sample are collected.

GCMS analysis: This extract was subjected to GC-MS analysis. GC-MS technique was performed using GC Shimadzu QP2010 system. The column used was Elite 1 fused silica capillary column. Mass spectra were recorded under scan mode in the range of 40-1000 m/z.

Antidiabetic Activity

Alpha amylase enzyme inhibition assay: A starch solution (1% w/v) was prepared by stirring 1g starch in 100 ml of 20mM of phosphate buffer (pH 6.9) containing 6.7mM of sodium chloride. The enzyme solution was prepared by mixing 27.5 mg of porcine pancreatic amylase (PPA) in 100 ml of 20mM of phosphate buffer (PBS, pH 6.9) containing 6.7mM of sodium chloride. To 100µl of (20, 40, 80,100 µg/ml) plant extracts, 200 µl porcine pancreatic amylase was added and the mixture was incubated at 37ºC for 10min. To the reaction mixture, 100 µl (1%) starch solution was added and incubated at 37ºC for 10 min. The reaction was stopped by adding 200µl DNSA (1g of 3,5 Di Nitro Salicylic Acid, 30g of sodium potassium tartarate and 20 ml of 2N sodium hydroxide was added and made up to a final volume of 100 ml with distilled water) and kept it in a boiling water bath for 20 minutes. There action mixture diluted with 2.2 ml of water and absorbance was read at 540nm. For each concentration, blank tubes were prepared by replacing the enzyme solution with 200ìL in distilled water . Control, representing 100% enzyme activity was prepared in a similar manner, without extract. The experiments were repeated thrice using the same protocol.

Tyrosinase enzyme inhibition assay: Tyrosinase enzyme solution is prepared for 1mg/ml concentration in 10% PBS(phosphate buffer saline, PH 6.9). To Various concentration of seeds extract of methanol and ethanol was prepared (20, 40, 60, 80, 100µg/ml) in each test tube and methanol was added to make upto the volume. 2 ml of the tyrosine solution was added to the test tube. They were incubated for 10mins at room temperature and OD was measured at 490nm after the incubation period. The pale white colour is obtained. Control, representing 100% enzyme activity was prepared in a similar manner, without extract. The experiments were repeated twice using the same protocol.

Method for calculation of α-amylase and Tyrosinase inhibitory concentration (IC_{50}). The concentration of the plant extracts required to scavenge 50% of the radicals (IC_{50}) was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I %) was calculated by

I % = (Ac-As)/Ac X 100,

where Ac is the absorbance of the control and As is the absorbance of the sample. Valuesare presented as percentage ± standard error mean of duplicates.

Results

The seeds of *Syzygium cumini* are collected from the local market. They were cleaned to remove dirt and stones by hand picking. The seeds ware collected and washed with distilled water. Collected samples were sundried for 24hour. The dried seeds are made to fine powder by grinding them by using electrical grinder, the powder were stored in an airtight container to avoid

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contamination due to moisture. The significance of the plant were shown in Table 1.

Fig. 1: *Syzygium cumini*

Table 1 : Significance of Phyto-constituents

Phytochemical	Significance
Alkaloids	Cytotoxicity
Tannins	Antidiarrheal, Anti-hemostatic, Anti-microbial
Steroids	Antibacterial
Flavonoids	Anti-inflammatory, Anti-microbial, Anti-cancerous, Antioxidant
Terpenoids	Inducing growth regulating, Antimicrobial activity
Phenols	Anti-apoptosis, Antiaging, Anti-inflammation
Glycosides	Low blood pressure.

Extraction: The powdered seeds were weighed for 1g and the selected solvent was taken for 100ml. The mantle is switched on and kept at 55ºC and the Soxhlet apparatus is set. The solvent and sample are loaded to the thimble and the flask and are started to heat. The water flow in the condenseris kept constant so that it does not allow heat transfer to the condenser. The setup was maintained undisturbed for 5hrs till the complete solubilization of the sample was attained. The concentrated extracts were collected and the remaining solvents residues were evaporated.

Phytochemical analysis: The phytochemical active compounds of *S.cumini*wasquantitatively analyzed for seeds and the results are presented in Table 2. Among these Phytochemicals tannins, phenol, flavonoids are present in both the sample extracts alkaloids are present only in *S.cumini*extract were saponins, glycosides, terpenoids are absent in both the extract in the present study phytochemical screening for both the extracts showed significant indication about the presence of metabolites that are alkaloids, tannins , flavonoids, terpenoids, phenol and steroids were found to be present in extracts. These are detected phytochemical compounds are known to have beneficial importance in medicinal as well as physiological activities. In this manner isolating and identifying these bioactive compounds, new drugs can be formulated to treat various diseases and disorders.

Table 2: Results for Phytochemical Screening

Properties	*S.cumini* ethanol extract
Alkaloids	-
Flavonoids	+
Tannins	+
Saponins	-
Phenols	+
Glycosides	-
Terpenoids	+

Total phenol content: Phenolic compounds are predominantly distributed in plants and they have gained much attention, due to their antioxidant activity and free radical scavenging ability with potential benefits for human health. Natural antioxidants available in medicinal plants have been alternative source of synthetic antioxidants. Total Phenol Content (TPC) results indicated that the differences in TPC among *S.cumini*products were statistically significant. The phenol content of *S.cumini* was 0.78mg/10g of plant extract both

seed extract showed similarly equal phenol contributing to the medicinal uses. The standard curve with gallic acid for TPC determination.These results demonstrated that the total phenol content of *S.cumini*and *N.sativa*seeds were equal to the content found in referred papers.

Antioxidant assay: DPPH (1,1 -Diphenyl-2-picrylhydrazyl) is a suitable nitrogen centered free radical which has a unpaired valence electron at one atom of nitrogen bridge. Scavenging of DPPH free radical is one popular antioxidant assay. The decolorization assay that will measure the capacity of antioxidants to directly scavenge DPPH radicals by monitoring its absorbance using spectro photometer at wavelength of 517nm. DPPH is a stable radical showing a maximum absorbance at 517nm. In the DPPH assay, the antioxidant was able to reduce the stable radical DPPH to yellow coloreddiphenylpicrylhydrazone. The method is based on the reduction of DPPH in solvent in the presence of hydrogen donating antioxidant due to the formation of non-radical form DPPH hydrogen in the reaction. DPPH is usually used as a reagent to evaluate free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The disappearance of the DPPH radical absorption at 515nm by the action of antioxidants is taken as a measure of antioxidant activity. The scavenging effect of *S.cumini*on DPPH radical linearly increased as concentration increased from 20 to100µg/ml. The 50% inhibition concentration of *S.cumini*was 20.63µg/.

TLC and column chromatography: Thin layer chromatography profiling of methanol and ethanol extract of both the extract gave results that directing towards the presence of number of phytochemicals. Different RF was calculated and it was nearly equal to 0.8, the retention values are calculated by making use of the distance traveled by the solvent to the distance traveled by the solute. These values shows different phytochemicals in different solvent systems. This validation in RF values of the phytochemicals provides a very important clue in understanding of their polarity and helps in selection of appropriate solvent system

Table 3: DPPH analysis

S.No	Concentration (µg/ml)	Inhibition % for S.cumini at 517nm
1	20	48.47 0.25
2	40	63.48 0.15
3	50	64.37 0.17
4	60	67.74 0.18
5	80	84.38 0.03
6	100	93.5 0.03

for separation of pure compounds by column chromatography. Mixture of solvents with variable polarity in different ratio used for separation of pure compound from plant extract the selection of appropriate solvent system for the particular plant extract was achieved analyzing the retention factor values of compounds in different solvent system.

Column chromatography, column was eluted with methanol and ethanol and 5 elutes for S.cumini were collected. Elutes are tested for purity of compounds by performing TLC and selected elutes are given for GCMS analysis. Elute 4 for S.cumini is selected and compounds are identified by GCMS analysis which shows the amount of constituents present.

GCMS analysis: *S.cumini* GC -chromatogram has shown the presence of 9 major peaks have been determined. Each of the peaks in the chromatogram represented the signal created when a compound eluted from the gas chromatogram column into the detector. The x-axis showed the RT that is retention time and y-axis measured the intensity of the signal to quantify the component in the sample injected. As the individual compounds eluted from the chromatogram column, they entered the electron ionization detector, where they were bombarded with a stream of electrons causing them to break apart into fragments. The fragments obtained were actually charged ions with

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a certain mass/charge ratio obtained was calibrated from the graph obtained, which was called mass spectrum graph which is the fingerprint of a molecule. Before analyzing the extract using GCMS, The temperature of the oven was maintained at 100ºC. Helium gas was used as a carrier as well as an eluent. The flow rate of helium was set at 1ml/min. The electron gun of mass detector liberated electrons having energy of about 70ev. The column employed here for the separation of components was done. The component peaks was identified from the database of National Institute Standard and technology (NIST) mass spectral library.

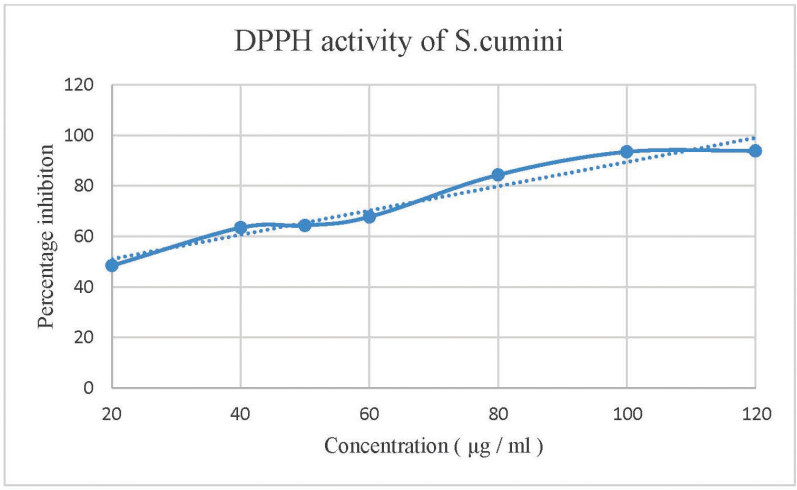

Fig. 2: DPPH curve for *S.cumini*

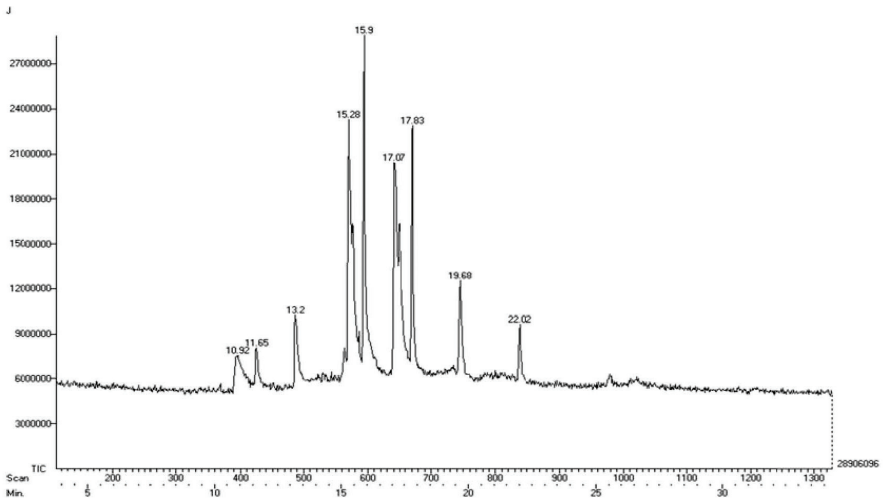

Fig. 3: GCMS results for *S.cumini* ethanol extract

Table 4 : Major peaks in *S.cumini* seed extract

S/N	Name	RT (min)	Formula	Molecular Weight
1	phenol	10.92	C6H5OH	191
2	Flavone	11.65	C15H10O2	222
3	2,4(1H,3H)-pyrimidinedione	13.2	C4H4N2	99
4	4H-1-Benzopyran-4-one	15.28	C9H6O2	268
5	Mitaflaxone	15.9	unknown	280
6	Coumarine	17.07	C9H6O2	163
7	4H-Benzopyran-4-one	17.83	C22H20N2O5	312
8	Quinoline	22.02	C9H7N	320
9	Benzofuran	19.68	C8H6O	162

Enzyme inhibitaion

Alpha amylase inhibition assay: In this study the *in vitro* a-amylase inhibitory activities of the methanol of S.cuminiwas investigated. The results of experiment showed that, there was a dose-dependent increase in percentage inhibitory activity against a-amylase enzyme. The methanol and ethanol extract (20-100µg/ml) of the plant exhibited potent amylase inhibitory activity in a dose dependent manner. Acarbose is a standard drug for a-amylase inhibitor. Acarbose concentration of (20-100 µg/ml) showed a-amylase inhibitory activity from 80µg/ml with an IC50 88.49µg/ml value at 1mg/ml concentration. A comparison of a-amylase inhibitory activity between the standard drug and plant extracts has been depicted. So the plant extract might be used as starch blockers since it prevents or slows the absorption of starch in to the body mainly by blocking the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides into maltose, maltose and other simple sugars. It can be achieved by retarding the absorption of glucose through the inhibition of carbohydrate digestion and prolong overall carbohydrate digestion time causing a marked decrease in the rate of glucose absorption thereby blunting the post prandial plasma glucose rise.

Table 5: a-amylase enzyme inhibition assay for the seed extract.

S.No	Concentration (µg/ml)	Inhibition % for *S.cumini* at 590 nm
1	20	46.6 0.75
2	40	48.0 0.77
3	60	49.3 0.79
4	80	50.6 0.81
5	100	51.8 0.83

Table 7: Tyrosinase enzyme inhibition assay for seed extract.

S.No	Concentration (µg/ml)	Inhibition % for *S.cumini* at 450 nm
1	20	14.2 0.18
2	40	19.0 0.17
3	60	28.5 0.15
4	80	42.8 0.12
5	100	57.1 0.09

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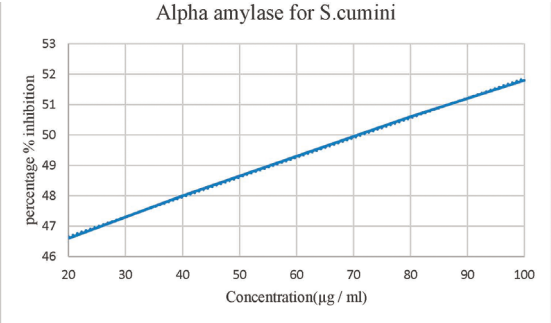

Fig. 4: α-amylase enzyme inhibition assay for *S.cumini* extract

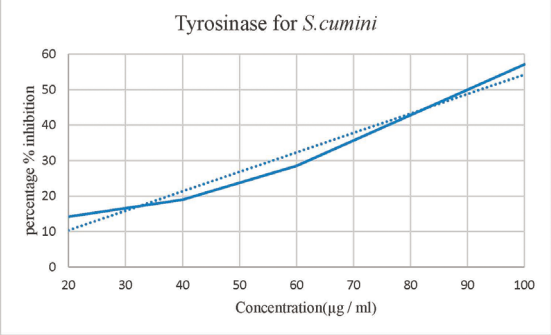

Fig. 5: Tyrosinase enzyme inhibition assay for *S.cumini* extract

Tyrosinase is a copper containing enzyme essential for tyrosine-melanin pigmentation. The role of toxic quinones in dopamine-induced neuronal damage and catalysing role in TYR in this process has been studied The data reveal that the seed extract are proven to inhibit the activity of the enzyme and are potential as neuroprotective agents via inhibition of Tyrosinase or TYR. The maximum inhibitory IC50 was 95.60 µg/ml for Black cumin. The combined extract inhibition of tyrosinase increased from 50 to 76% of its activity were it gave best results when they are combined. Thus the extracts showed the better source for anti-hyper pigmentation or neuroprotective agents. The activity increased the inhibition of tyrosinase when the combined polyphenols from S.cumini extracts.

Discussion

Many herbal extracts have been reported to have antidiabetic activities and are used for in Ayurveda for the treatment of diabetes[2,4,9]. Herbal extracts have been used directly and indirectly for the preparation of many modern medicines. In this study, an in vitro inhibitory effect of Syzygium cumini extracts polyphenol effect on porcine pancreatic amylase activities was evaluated.

Antidiabetic and Neuroprotective activities of extracts have been reported with the seeds of S.cumini. Raza et al., [3] examined the inhibition kinetics of acarbose and its two analogues, on alpha amylase from porcine pancreas.They showed the similar type of inhibition for S.cumini seed extract as evidenced by the Dixon was obtained according to K. Karthic et al., [10].

Tyrosinase inhibitory activity of different plant extracts has been reported and tyrosinase inhibitors have been tested in cosmetics and pharmaceuticals as a way of preventing over production of melanin in epidermal layer. Tyrosinasecatalases the oxidation of L-tyrosine to L-DOPA and L-DOPA to dopaquinone. These reactions are the initial steps of melanin biosynthesis in the course of searching for anti-hyper pigmenting agents. Spices are used extensively in the Indian food habits irrespective of the region or state they belong [12].

This study proves the efficacy of these seeds to be more useful in cosmetics as it is mostly used in skin care preparation for its property of promoting skin care health and to improve its complexion. Thus, this investigation led us to search for a naturally occurring tyrosinase and amylase inhibitors from Indian traditional medicine, which can further be exploited for its possibly responsible phytoconstituents[17].

Antidiabetic activity of different fruit parts of S.cumini are measured for a-amylase inhibition assay the results showed that the methanolic extract has significant variation in antidiabetic activity. The kernel parts among fruit has higher

antidiabetic effect and the seed has the next higher effect with difference of 10% increase in inhibition activity against enzyme alpha amylase. The lower the IC50 value indicates the higher the inhibition activity. Itis proved that the seed of S.cumini has the potential 70% inhibition against alpha amylase enzyme at minimum concentration

The polyphenol compounds that are not isolated will also have the potential to inhibit the activity of tyrosinase and amylase enzyme by both the seed extracts.

The S.cumini seeds and fruits have shown antioxidant and anticancer activity. The ethanol extract has highest antioxidant that is free radical scavenging activity and anticancer property. The ethanol extract have shown equal result when compared with the previous studies. The antioxidant activity of S.cumini has more than 80% scavenging activity.

The polyphenolic bioactive compounds present showed major enzyme inhibition that is Amylase and Tyrosinase activity which possess diabetes and neuronal diseases, inhibition of the activity may show potential source of antidiabetic and neuroprotective source. Individual activity is seen to be efficient and the combined activity of these two extracts that is the combined activity of the compounds in the seeds showed increase in their activity and increase therapeutic potential. According to the inhibitory activities of the two main group of enzymes, one group in relation with Neurotransmission metabolism (TYR), whereas other tested is current targets for antidiabetic drugs (a-amylase). S.cumini extracts were able to inhibit TYR and a-AMY enzymes, they have been described to be involved in inhibition, which may result in an anti-depressed and anxiolytic effect. In the conclusion, the work revealed that S.cumini are potential useful natural products to employ in the prevention of certain diseases in which oxidative stress may be major etiological role player. Both extracts are the best source of polyphenols with potential effects as neuro-protective and anti-hyperglycemic agents.

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Current Trends in Biotechnology and Pharmacy
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Autologous Stem Cell Transplantation to Treat Emery- Dreifuss Muscular Dystrophy

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Abstract

Emery- Dreifuss muscular dystrophy affects muscles such as Skeletal muscles, Cardiac muscles which is named after Eglin H. Emery and Fritz Emery- Dreifuss. Here we are going to demonstrate Autologous Stem cell transplantation done in our hospital to treat this dystrophy and discuss about post transplantation follow up out come

Key words: Muscular dystrophy, Emery-Dreifuss, Skeletal muscle, Cardiac muscle.

Introduction

Emery- Dreifuss muscular dystrophy is a condition that affects skeletal muscles and cardiac muscle. It is caused by mutation. This is x- linked autosomal dominant or autosomal dominant recessive fashion. The mutation are caused in Emery- Dreifuss muscular dystrophy and LMNA, FHLI genes. Joint symptoms are present in child hood and involve contractures of neck,elbows ankles ,children and adults with this dystrophy usually experience slowly worsening of muscle wasting and muscle weakness. By adulthood many people with this type of dystrophy develop cardiac problems such as arrhythmias and conduction defects. Here, we are going to demonstrate a male boy with age 18 who has muscle weakness in upper arms and lower legs and hips. He has elevated serum creatine phosphokinase levels with 2000 U/L and myotonia and pectus excavatum and a detailed explanation about autologous stemcell therapy was given to him and his parents with its pros and cons. A written consent was obtained from the patient and his parents to proceed for autologous stemcell therapy .Preoperative blood investigations and cardiac checkup was with in normal limits and the patient was kept Nill by mouth 6hrs before the procedure to ensure that he will be not as aspirated during general anesthesia. Under general anesthesia 100ml bone marrow was aspirated and 126 $x10^6$. Autologous stemcell were isolated under sterile conditions and were injected into all affected muscles deeply. Cardiac muscles were excluded because he didn't develop any cardiac manifestation. For cardiac muscles it is standardized protocol to inject autologous stemcells into coronary artery .The patient was recovered from anesthesia and he was kept on antibiotics and anti inflammatory medication for 10 days. On the next day he was discharged from the hospital as there were no post operative complications. He was followed for 2 yrs for every 15 days as his upper and lower limb power was improved gradually.

Conclusion

This autologous stemcell transplantation is a milestone in medical field particularly in neurological disorders especially in this Emery-Dreifuss muscular dystrophy.

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The Implication of a Novel Herbal Formulation in Reversal of Drug-Resistance for Cancer Treatment

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Abstract

Traditional medicines or herbal medicinal compounds are being increasingly considered as useful complementary and alternative treatments for cancer. A large number of *in vitro* and *in vivo* studies have reported the beneficial effects of herbal medicines alone and in combination with conventional therapeutics. We developed a poly-herbal formulation Swastharakshak® (SR033), exhibiting anti-tumorigenic property. Although, majorly 5-Fluorouracil (5FU) resulted in necrosis of HeLa cells, however in combination with SR033, the majority of cells underwent apoptosis with an increase of 30%, SR033 alone was considered as an experimental control. Moreover, 5FU and PTX resistance were reversed by SR033 with $2.5\times10^3$and 0.17×10^3 fold, respectively, in resistant HeLa-R cells. We observed a 2 fold decrease in free radicals when treated with SR033 as compared to untreated controls; however, 1.2 fold decrease was observed in SR033 + 5FU groups in comparison to 5FU alone.These results demonstrated that SR033 acts synergistically with 5FU, along with chemo-protective and 5FU resistance reversal property. Hence, SR033 is a potential herbal formulation that could be used effectively with 5FU as a combination therapy for cancer.

Key words: Herbal medicine, Cancer, Drug-resistant cell line, 5-Fluorouracil, Integrative therapy, Drug Resistance Reversal.

Introduction

Cancer is one of the most common non-communicable diseases and a leading cause of death worldwide. It is a second deadly disease with an estimate of 9.6 million deaths worldwide in 2018 (1). Moreover, low- and middle-income countries are at higher risk and they count approximately 70% of the cancer deaths (1). Even though chemotherapy is central to clinical management of cancer, failure in chemotherapy is not uncommon, mainly due to the dose-limiting toxicities, which is also associated with the occurrence of drug resistance. Apart from the modern technologies such as the use of nanoparticles, dendrimers to deliver chemo drug, use of natural compounds/ products to use as adjuvant enhancing chemo drug activity and to counteract drug resistance may be beneficial (2).

Cancer cells have increased levels of oxidative stress as compared to their normal counterparts. Maintaining reactive oxygen species (ROS) homeostasis is crucial as it promotes cell proliferation and differentiation at a moderate level (3, 4), whereas causes oxidative damage at higher levels (5). Currently, 313 drugs have been approved by the Food and Drug Administration to treat cancer. At least 40% of them can induce oxidative stress (6) due to their non-selective nature. Therefore, manipulating ROS levels by redox modulation is a way to selectively kill cancer cells without causing significant toxicity to normal

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cells (7) by the presence of some exogenous factors, such as drugs, radiations and diet (8). ROS mediated mechanisms also provide resistance towards many anti-cancer agents (9). Additionally, the generation of endogenous ROS in combination with the drugs which initially induce ROS production may contribute to a decrease in the sensitivity to these drugs in long-term treated cancer cells (10-12). Due to these reasons, the survival rate of cancer patients is not significantly encouraging. To face this complex situation, many researchers are turning to find out an alternative treatment plan which can be used as therapeutics, as an adjuvant treatment besides other treatments, or as chemo-preventive agents (13-16), that can avoid and/or minimize the risks of unusual side effects and improve the efficiency of conventional treatment approaches (17). This constitutes a major step towards cancer management, which is holistic and comes under the ambit of integrative oncology. Various herbal compounds and their formulations are being used for effective cancer treatment, among these, epigallocatechingallate (EGCG) and curcumin are widely used.

Epigallocatechingallate, one of the most phenolic catechins present in green tea, exhibit chemo-protective and chemo-preventive action against various cell lines (18) by inhibiting the interaction of tumor promoters (ligands) with their receptors on the cell membrane, known as “Sealing effects of EGCG” (19). It also acts synergistically with anti-cancer drugs in tertiary cancer prevention (20-23). Furthermore, in combinational therapy, the presence of EGCG significantly increased the bioavailability of tamoxifen (24), 5-fluorouracil (25), and doxorubicin (26) and also showed synergistic enhancement of anti-cancer activity against human cancer cell lines (27-29). Moreover, it is suggested that from clinical trial based studies of breast cancer, EGCG shows protection against the toxic effects of chemotherapy (30). Another compound, curcumin is a polyphenol found in turmeric has broad pharmacological activities, including anti-inflammatory, anti-oxidative, and anti-tumour effects (31). Also, it has been found that curcumin can reverse drug resistance in tumour cells from gastric, hepatic and cervical cancers as well as other malignancies (32,33).

We have developed a poly-herbal formulation, SR033 and have been tested against 5FU sensitive HeLa and resistant (HeLa-R) cell line. SR033 (patent applied, FSSAI approved manufactured by Sri Raghavendra Bio-technologies Pvt Ltd, Bangalore) is a composition of five herbal compounds which are well established anti-oxidative, anti-cancer, chemo-preventive, chemo-protective, anti-proliferative and anti-inflammatory (34-38). The formulation shows chemo-protective, chemo-sensitization, apoptotic and anti-oxidative properties against cancer cell lines.

Materials and methods

Maintenance of cells: Human cervical cancer cell line HeLa (drug-sensitive) was obtained from NCCS, Pune. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher) in a humidified incubator at 37ÚC with 5% CO_2. Trypsin digestion with 0.25% trypsin solution was used for cell passaging. 5FU sensitive and resistant HeLa cells (HeLa and HeLa-R, respectively) were continuously cultured in 0 µg/mL and 2 µg/mL of the drug, respectively. *In vitro* experiments were carried out at 80% cell confluency and confirmed in at least three independent experiments, each performed in triplicates.

Development of resistant cells: Stably resistant cells to 5-Fluorouracil (5FU) were developed in our laboratory, as described earlier(39) with slight modifications.HeLa cells were exposed to an initial dose of 0.1 µg/mL 5FU and surviving cells were cultured for 10 days and two passages. The cells were exposed to increasing concentrations of 5 FU to 0.5 µg/mL, 1.0 µg/mL and 2.0 µg/mL (40). The surviving resistant cells were named as HeLa-R. These cells were also tested for paclitaxel (PTX) resistance.

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Detection of cytotoxic effects of SR033, 5FU and PTX on HeLa and drug-resistant HeLa-R cells: Single-cell suspensions of HeLa and HeLa-R cells were prepared in DMEM medium at log phase of growth. Each well of 96-wells plate was seeded with 100 µL/well (2×10^4 cell/mL) of the cell suspension, and the plate was incubated for 24 h to allow cell adhesion. Different concentrations of Swastharkshak® (SR033) ranging 0, 20, 10, 5, 2.5, 0.625, 0.31, 0.1 mg/mL SR033 (100 µL in each well) were added to drug-sensitive and drug-resistant cells for 24 h and 48 h. Similarly, 5FU was added to HeLa and HeLa-R cell lines at different concentrations, ranging 0.015, 0.031, 0.062, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 µg/mL and 53, 106, 312, 625, 1250, 2500, 5000 µg/mL, respectively and PTX was added at concentrations 1.57, 3.13, 6.25, 12.5, 25.0, 50.0 $\times10^{-3}$ µg/mLto both the cell lines. These drugs were added individually to both cell lines to determine their IC_{50} value. The culture medium was aspirated and fresh medium was added with MTT dye to respective wells and incubated for 4 h. The complete solution was aspirated and DMSO was added to dissolve Formazan crystals. The plate was incubated for 30 minutes and values were measured using a microplate reader at OD_{545}. The IC_{50} of SR033 was calculated. IC_{50} value determines the concentration of drug at which 50% of cells survive. Drug Resistance Index (DRI) of 5FU on HeLa-R cells was calculated using the formula: IC_{50} of resistant cells/ IC_{50} of sensitive cells (41).

Determination of apoptosis using Hoechst staining: Hoechst staining was performed for qualitative and quantitative analysis of apoptosis in HeLa cells with SR033 alone, 5FU alone and SR033 in combination with 5FU. HeLa cell suspension (0.3×10^6 cells) was cultured in 35 mm dishes for 24 h to allow cell adhesion. Cells in each dish were treated with 2 mL DMEM culture medium (supplemented with 10% FBS), containing medium alone, SR033 (IC_{50}), 5FU (IC_{50}) and SR033 (IC_{50}) + 5FU (IC_{50}). After cells were cultured for 48 h, the culture medium was aspirated and cells were washed with 1X Phosphate buffered saline (PBS). Cells were then trypsinized, the pellets were spread and air dried completely on grease-free slides. The slides were treated with paraformaldehyde (4%, pH=7.4) prepared in 1X PBS for 30 minutes and then dipped in a coupling jar containing 1X PBS, three times. Each time fresh PBS was added to coupling jars. The slides were dried and Hoechst stain was added in the dark (42). The stain was further washed by using 1X PBS and slides were observed using a fluorescence microscope.

Drug resistance reversal effect of SR033 on drug-resistant HeLa-R cells: Single cell suspension of HeLa-R cells in log phase growth was prepared in DMEM and incubated in 96 wells plate (100 µL/well at 2×10^4 cells/mL) for 24 h to allow cell adhesion. Cells were treated with 600 µg/mL SR033 (100 µL) and different concentrations of 5FU (4, 2, 1, 0.5, 0.25, 0.12, 0.06, 0.03, 0.01 µg/mL) along with untreated controls. After cells were cultured for 48 h, the culture medium was aspirated and fresh medium with MTT reagent (5 µg/mL, 100 µL for each well) was added to each well. The plate was then incubated for 4 h. The complete solution was aspirated and DMSO was added to dissolve Formazan crystals. The plate was incubated for 30 minutes and values were measured using a microplate reader at OD_{545}.The drug resistance reversal ratio was calculated according to the following formula: IC_{50} in blank control group/ IC_{50} in the reversed group (43). Similarly, the experiment was performed to determine the drug reversal effect by SR033 (600 µg/mL) with respect to Paclitaxel (0.04, 0.08, 0.17, 0.35, 0.70, 1.41, 2.83, 5.66 $\times10^{-3}$ µg/mL).

Determination of Superoxide Dismutase (SOD) in HeLa-R cells : HeLa-R cells were seeded in 35 mm dishes and treated with IC_{50} values of SR033 and 5FU in different groups after 24 h incubation. Cells were trypsinized and the lysate was prepared by using RIPA lysis buffer (Thermo Scientific) (44).The supernatant was collected and the assay was performed using Cayman's Superoxide Dismutase detection kit (45). The per

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cent inhibition was plotted as a function of final SOD activity (U/mL). The unknown samples' absorbance was later mapped to its corresponding SOD activity using the formula, Percent inhibition by SOD= [(control absorbance - sample absorbance)/ control absorbance]×100, where one unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The values were interpreted in terms of percent free radicals.

Statistical analysis: GraphPad Prism 5.0 was used to analyze and represent graphical data. One way ANOVA was performed, followed by Turkey's test. The experimental data are expressed as mean and standard deviation. Statistical significance was achieved when $p<0.05$.

Results and Discussion

Cytotoxic effect of SR033, 5FU and PTX on HeLa and drug-resistant HeLa-R cells: To understand the cytotoxic effect of SR033 on HeLa and HeLa-R cells, MTT assay was performed and the following analysis was undertaken at different concentrations and time points. IC_{50} of SR033 on HeLa cells was 500 µg/mL at 24 h and 600 µg/mL at 48 h. The IC_{50} of SR033 on HeLa and HeLa-R cells at 24 h was 500 µg/mL and 1200 µg/mL, respectively (Figure 1A and B). IC_{50} of 5FU and PTX was also determined by MTT assay to calculate DRI of HeLa-R. The IC_{50} of 5FU on HeLa and HeLa-R cells was 2 µg/mL and 5000 µg/mL (Figure 1C), respectively, with DRI of 2.5×10^{3}. The IC_{50} of PTX on HeLa and HeLa-R cells was 5.96×10^{-3} µg/mL(Figure 1D) and 13.430×10^{-3} µg/mL, respectively, with DRI of 2.25.In several studies, IC_{50} of 5FU ranges from 2-5 µg/mL on various cancer cell lines; moreover this dose of 5FU is not effective on 5FU resistant cells (43,46,47). IC_{50} values of 5FU on a colon cancer cell line, HT-29 was exceptionally higher (39 µg/mL) compared to other cell lines[48].In a recent study, it was reported that DRI of HCT-8 cells is 74.12(43). DRI obtained in this study is significantly higher than that reported earlier, higher DRI would be impactful for testing the effectiveness of any chemo-sensitizing compound. This study also reveals the multi-drug resistance of HeLa-R cells, as cells were also found resistant to PTX, in addition to 5FU. IC_{50} values obtained in this experiment were used to further determine drug resistance reversal by SR033.

Synergistic effect of SR033 and 5FU on apoptosis of HeLa cells: Apoptosis is programmed cell death and is programmed to occur in a sequentially with minimal inflammation or injury to the surroundings. Chemo-drug kills cancer cells by necrosis which also damages the nearby cells/ tissues(43,49), Curcumin and green tea extract inhibit cancer cell proliferation (49-52), eventually, cancer cell leads to apoptosis. In this study, apoptosis was qualitatively and quantitatively analyzed using Hoechst stain. Cell morphology in treated groups has been compared with media control (Figure 2A). Cells were considered necrotic and apoptotic on the basis of nuclear structure and its stain intensity. Cellular membrane was ruptured in necrotic cells, whereas was intact and brightly stained nucleus was observed in living cells. Blebs were formed and brightly stained fragmented DNA was observed in apoptotic cells. SR033 alone and 5FU alone groups are comparable with SR033 along with 5FU treated group. Cells treated with 5FU alone underwent necrosis. Cells treated with SR033 alone showed 45.9% apoptosis, whereas combination with 5FU showed 80%, which is a 30% increase in apoptosis rate (Figure 2B), showing its synergistic effect.Similar studies were performed in HCT-8 and EC9706 cells, where 15% and 20% increase in apoptosis was noticed when treated with the combination of curcumin and 5FU (43,46). SR033 showed a remarkable increase in apoptosis rate when compared with the above-reported data (43,46).

Drug resistance reversal effect of SR033 on resistant HeLa-R cells : Chemo-drugs like 5FU lead to resistance in cancer cells due to continuous dosage. Curcumin and green tea extract are known to exhibit drug resistance reversal properties (49-52). When 600 µg/mL SR033 (IC_{50} on HeLa) was added to HeLa-R with

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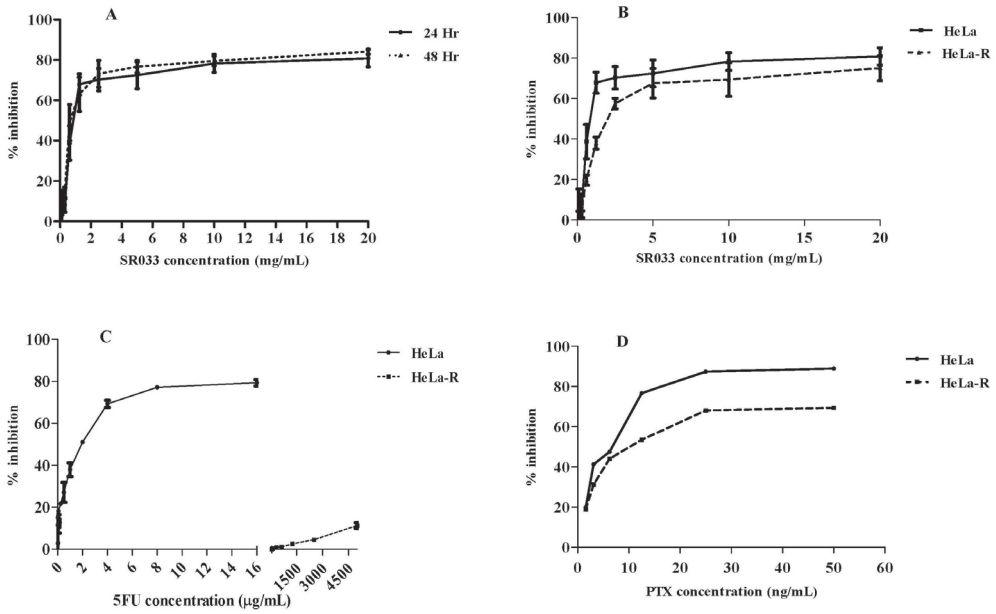

Fig. 1. Cytotoxicity assay for SR033, 5FU and PTX on HeLa and HeLa-R cells. A. Effect of SR033 on HeLa cells. B. Effect of SR033 on HeLa and HeLa-R cells. C. Effect of 5FU on HeLa and HeLa-R cells. D. Effect of PTX on HeLa and HeLa-R cells.

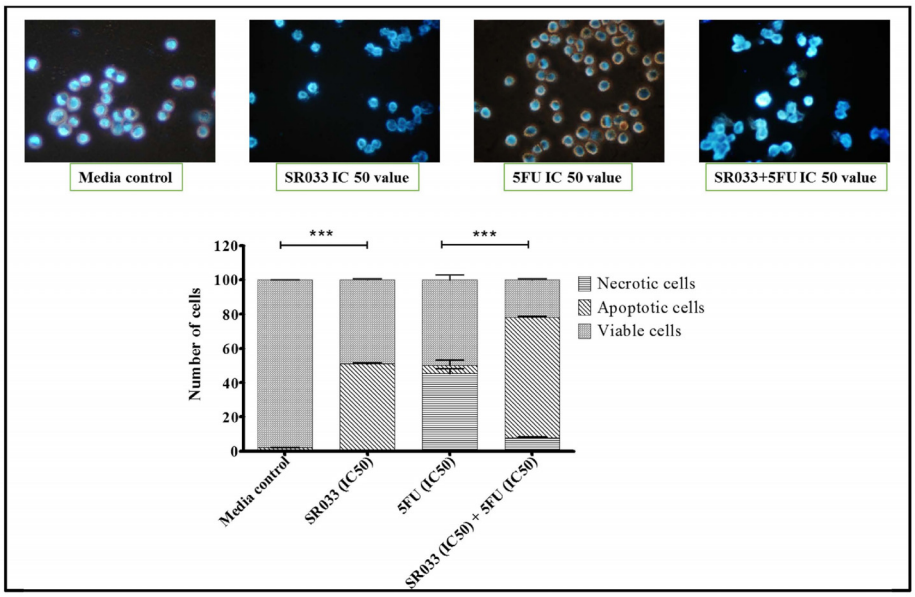

Fig. 2. Synergistic effect of SR033 and 5FU on HeLa cells. Hoechst staining of HeLa cells in different growth conditions (above panel). Below is the bar graph showing quantitative analysis of dead cells including apoptotic and necrotic cells and viable cells (cumulative result obtained from 10 fields of each group). $p<0.001$ (triple star) when compared media control with SR033 and 5FU with combination of SR033+5FU.

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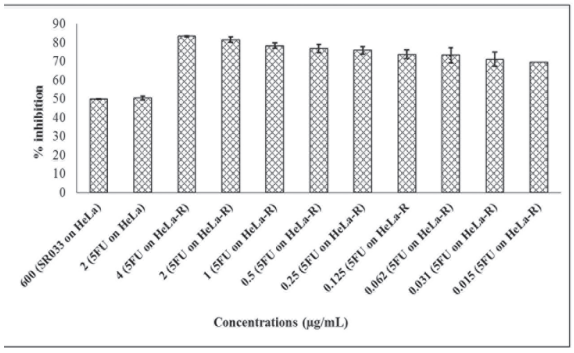

Fig. 3A. Drug reversal property of SR033 on resistant HeLa-R cells. SR033 and 5FU were used alone at IC50 obtained from HeLa cells. Drug reversal effect was observed with SR033 IC50 on HeLa in combination at various doses of 5FU below IC50.

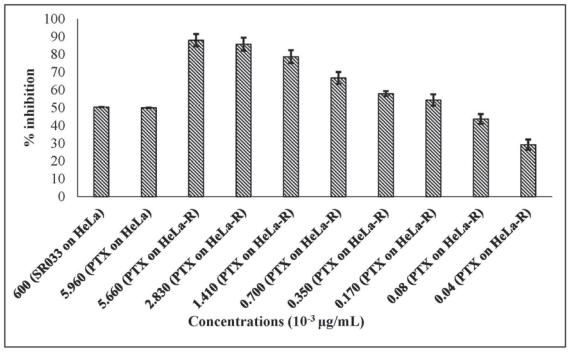

Fig. 3B. Drug reversal property of SR033 on resistant HeLa-R cells. SR033 and PTX were used alone at IC50 obtained from HeLa cells. Drug reversal effect was observed with SR033 IC50 on HeLa in combination at various doses of PTX below IC50.

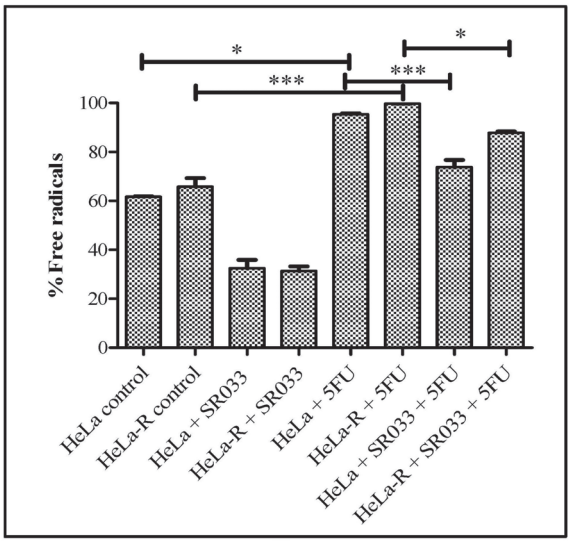

Fig. 4. Effect of SR033 on percent free radicals produced in combination with chemo-drug, 5FU. $p<0.001$ as shown with triple asterisk (***) and $p<0.05$ shown as single asterisk (*).

various low concentrations of 5FU, the inhibitory effect of SR033 + 5FU was significantly high. Treatment of HeLa-R cells at IC50 values of HeLa cells in the combination of SR033 (600 µg/mL) with 5FU (2 µg/mL) and PTX (5.96×10^{-3} µg/mL) resulted in 80% and 88% death of HeLa-R, respectively (Figure 3A, 3B). It is noteworthy to achieve $2.5\times10^{3}$and 0.17×10^{3} fold drug resistance reversal to 5FU and PTX, respectively. In earlier studies, 2 and 3.71 fold reversal of 5FU has been shown in resistant HCT116 and HCT-8 cells by curcumin, respectively (43, 53) Although, a study by Tian*et al* (2012) could not show significant 5FU resistance reversal in Esophageal squamous cell carcinoma by curcumin (54). The reversal ratio with SR033 was comparatively higher when observed with the above-reported data of curcumin (43, 53).

SR033 increases SOD in HeLa and HeLa-R cells: Superoxide Dismutase was analyzed for the combination of SR033 and 5FU to reveal the chemo-protective action of SR033. A high SOD value indicates the lower oxidative stress in the sample, i.e. the SOD activity (U/ml) will be higher if free radicals have been suppressed (55) and the higher per cent inhibition by SOD enzyme signifies lower oxidative stress and free radicals. The results obtained show significantly low free radicals for both HeLa and HeLa-R cells when treated with SR033 (Figure 4). In effect, the study showed that the usage of 5FU drug alone generates free radicals. The increase in free radicals in 5FU groups has been significantly ($p<0.001$) subsided by SR033 in the groups

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treated with SR033 + 5FU, hence displaying its antioxidant capability. However, in earlier reports, use of 5FU with Irinotecan resulted in a 3 fold decrease in free radicals (56), whereas no significant increase was observed withbetulin-3, 28-diphosphate (57). In this study, we observed 2 fold decrease in free radicals when treated with SR033 as compared to untreated controls, however, 1.2 fold decrease was observed when compared 5FU alone groups with SR033 + 5FU groups. The effect of SR033 in combination to 5FU showed comparable results to above-mentioned reports (56,57). While the data does show that SR033 alone significantly reduces the number of free radicals, the practical application of using SR033 alone to treat cancer is not feasible (58).Similar studies were also performed earlier (59,60).

Conclusion

Since 5FU is used as one of the common chemo-drugs for cancer treatment, but toxicity and resistance is the major drawback of this drug (61,62). Nowadays, application of herbal compounds as complementary and alternative medicine/ adjuvants is increasing to overcome chemo-toxicity and resistance. Thus, the combinatorial approach with herbal compounds can be adopted to reduce the toxic effects of chemotherapy as well as combating drug resistance (50-54,56-60). In the present study, we have tested a poly-herbal formulation, SR033in combination with 5FU to reduce toxicity, to increase its efficiency and to prevent oxidative damage. Our study revealed a reversal of $2.5\times10^3$and 0.17×10^3 folds chemo-resistance by the synergistic effect of SR033 with 5FU and PTX, respectively, where $IC_{80}$and IC_{88} has been achieved with IC_{50} values by combining SR033 with 5FU and PTX, respectively, which implies that SR033 increases the efficacy of the drug at lower doses. Also, SR033 decreases the oxidative stress thus averting the collateral damage caused due to chemotherapy. Thus, the study concludes SR033 as a strong candidate for cancer treatment in combinational therapy with 5FU.

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Optimization of Process Parameters for High Yield Production of Exo – Inulinase from *Trichoderma asperellum* RSBR08 by Using Solid State Fermentation

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Abstract

Inulinase is an industrially applicable enzyme which is used widely in the production of ultra high fructose syrups and fructo-oligosaccharides. Production of inulinase enzyme in a cost effective manner is the major challenge faced. In the present research study, *Trichoderma asperellum* RSBR08 which has the capability to produce high exo-inulinase enzyme is produced by using the solid state fermentation. Process parameters for production of exo-inulinase by using solid state fermentation were optimized. Results showed high exo-inulinase production in garlic as substrate (199.2±6.3 U/gds), 45% moisture percentage (228.4±3.4), 26°C temperature (219.1±5.8) and 5.0 pH (213.6±4.5). Effect of different metals *viz.* Mg^{2+},Zn^{2+}, K^{+},Ca^{2+}, Na^{+},Mn^{2+} and Hg^{2+} were studied on the production of exo-inulinase enzyme in which Ca^{2+} induced the enzyme production (196.3±5.6) whereas Hg^{2+} has inhibited the inulinase production (37.0±3.3). Based on the above results, the solid substrate fermentation parameters optimized can be used industrially.

Key words: *Exo-inulinase, Metal ions, Solid state fermentation, Trichoderma asperellum*

Introduction

Inulin is a linear polymer of fructose with b, 2-1 linkage terminated with glucose unit by a sucrose type linkage at the reducing end (1). Inulin is available abundantly in roots and tubers of garlic, jerusalem artichoke, chicory, dandelion, onion, leek and dahlia (2). Inulin is an inexpensive and readily available substrate for the production of fructose syrups and fructo-oligosaccharides. Fructose is much preferred now-a-days because of its sweetening property and insignificant insulogenic effects (3).

Fructose units can be liberated from inulin by the regular acid hydrolysis process which will be carried out at temperature 80 - 90°C and 1 - 2 pH. But the acid hydrolysis process results in the degradation of fructose and formation of di-fructose anhydrides which results in the formation of colored end product. Due to these drawbacks of acid hydrolysis process, inulinase enzymes gained much importance by which 90 - 95% of fructose can be recovered in a single step without formation of any bi products (4, 5).

Initially, inulinases were isolated from plant sources but due to its less productivity, microbial inulinases were much focused (6). Bacteria, yeast and fungal strains which produces inulinases are *Aspergillus spp., Pencillium spp., Xanthomonas spp.*, *Fusarium oxysporum, Rhizopus spp., Streptomyces spp., Acetobacter spp., Artrobacter sp, Bacillus spp, Schizosacchromyces alluvius, Candida spp., Trichoderma spp., Pseudomonas spp.*, *Kluyveromyces spp.*,*Cryptococcus spp.*, *Pichia spp.*, *Sporotrichum spp.*(7, 8, 9).

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Inulinases are broadly categorized into 2 types based on the catalytic activity on inulin *i.e.* exo-inulinase and endo-inulinase. Exo-inulinase (â-2-1-D-fructan fructohydrolase) hydrolyses and liberates the individual fructose units from non-reducing end where as endoinulinase (â-2-1-D-fructan fructanohydrolase) hydrolyses randomly at the internal linkages of inulin (10).

Inulinases can be produced by both submerged and solid state fermentation. But solid state fermentation has gained much importance because of its high yield, operation ease, high product recovery and cost effectiveness (3, 11). In solid state fermentation, selection of substrate plays a major role which is core for enzymatic process. An appropriate substrate selected needs to be cost effective and readily available. In the current study, various plant materials rich in inulin and cost effective were selected for the production of inulinase (12).

In the current research work, substrate and process optimization studies were carried out for high yield production of exo-inulinase from *Trichoderma asperellum* RSBR08 using solid state fermentation.

Materials and Methods

Chemicals and reagents: All the chemicals and reagents were purchased from Fisher scientific, Mumbai, India.

Sourcing of inulinase producing fungal strain: Exo-inulinase producing *Trichoderma asperellum* RSBR08 was sourced from culture collection of R&D center, SOM Phytopharma (India) Limited, Hyderabad, India. The fungal culture was subcultured on potato dextrose agar slants and stored at 4°C.

Substrate materials collection and preparation: Chicory, jerusalem artichoke, garlic, onion were procured from vegetable market in Kukatpally, Telangana, India (17.4948°N, 78.3996°E). Wheat bran, coconut oil cakes were purchased from the local market of Suraram, Telangana, India (17.5412°N, 78.4338°E). Sugarcane bagasse was collected from sugarcane crushing shops in Miyapur, Telangana, India (17.5169°N, 78.3428°E). Substrates collected were washed under running tap water and chopped into small pieces. Chopped pieces were dried in hot air oven at 80°C for 24 h. The pretreated substrates were used for solid state fermentation.

Inoculum preparation : Exo-inulinase producing *Trichoderma asperellum* RSBR08 was inoculated in liquid medium with the composition g/L: inulin – 10 g/L, yeast extract – 10 g/L, $NaNO_3$ – 10 g/L, $KH\ PO_4$ – 5 g/L, $MgSO_4.7H_2O$ – 1 g/L, pH – 5.0. Inoculated flask was kept on orbital shaker at 120 rpm for 96 h (12).

Solid state fermentation: Solid state fermentation was carried out in HDPE autoclavable bags. 150 g of each substrate was taken in each bag and 40% moisture was maintained with the minimal media composition (g/L - yeast extract – 1 g/L, $NaNO_3$ – 1 g/L, $KH\ PO_4$ – 0.5 g/L, $MgSO_4.\ 7H_2O$ – 0.1 g/L). Substrate bags were autoclaved at 121°C and 15 psi for 30 min. $1.0x10^8$ fungal spores were added in each bag, mixed thoroughly and incubated at 28°C for 96 h (7). Substrate showing high inulinase activity was used for further optimization studies.

Analysis of inulinase activity : Inulinase assay was performed for the amount of fructose units liberated from the inulin. Reaction mixture with 0.5 ml enzyme extract, 0.5 ml of 1% (w/v) inulin in 0.2 M sodium acetate buffer with pH – 5.0 and incubated at 50°C for 15 min. Amount of reducing sugars liberated were measured by using Somogyi copper reagent and absorbance was taken at 520 nm. Inulinase activity can be calculated as the amount of enzyme liberated 1 µmol of fructose per minute under the assay conditions and expressed as units of activity per gram solid substrate (U/gds) (3).

Optimization of parameters in solid state fermentation: Substrate showing high inulinase activity was used further for solid state fermentation optimization studies.

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Moisture percentage: Moisture percentage in the solid state bags was maintained by minimal nutrient solution. Different moisture percentages *i.e.* 25, 30, 35, 40, 45, 50, 55 and 60 were maintained in different solid state bags. Bags were inoculated with *T. asperellum* RSBR08 culture and incubated at 28°C for 96 h. Inulinase assay was carried out by using the standard protocol.

Temperature: Different temperatures ranging from 20°C - 30°C (20, 21, 22, 23, 24, 25, 26, 27, 28, 29 and 30°C) were maintained. Substrate bags were inoculated with the *T.asperellum* culture and incubated at different temperatures for 96 h. Samples were drawn after the incubation period and inulinase assay was carried.

pH: In solid state fermentation, pH of the substrate bags was maintained by changing the pH of the minimal nutrient solution. pH ranging from 3-8 was maintained in different bags and inoculated with the fungal culture inoculum. Inoculated bags were incubated at 28°C for 96 h and assay was carried out.

Effect of metal ions on inulinase production: Effect of metals on inulinase activity was measured by using $MgSO_4$, $ZnCl_2$, KCl, $CaCl_2$, NaCl, $HgCl_2$ and $MnSO_4$ with a concentration of 0.1% w/v. Each substrate bag is inoculated with different metal and autoclaved. Sterilized substrate bags were inoculated with fungal culture and incubated at optimum conditions (13).

Results and Discussion

Increase in demand for inulinases and applicability in the production of fructo-oligosaccharides, inulio-oligosaccharides, and high fructose syrups *etc* created inquisitiveness to search for new inulinase sources. In this scenario, microbes have grabbed much attention with their ability to produce high inulinase yields and ease of production (14). In the current research study, attempts were made to optimize the process parameters for exo-inulinase production by using *Trichoderma asperellum* RSBR08 by solid state fermentation technology.

Potent exo-inulinase producing *Trichoderma asperellum* RSBR08 was sourced from culture collection of R&D center SOM Phytopharma (India) Limited, Hyderabad. For substrate optimization studies several substrates *viz.* Chicory, jerusalem artichoke, garlic, onion, wheat bran, coconut oil cake and sugarcane bagasse were collected and pretreated. 40% of moisture content was maintained with the minimal medium, bags were autoclaved and inoculated with the *T.asperellum* culture. After 96 h, the substrate samples were collected and inulinase activity was checked. Among all the substrates Garlic showed high exo-inulinase activity (199.2±6.3 U/gds) followed by Jerusalem artichoke (169.9±5.2 U/gds) and Chickory (157.6±5.7 U/gds). Fig. 1 Similar research reports showed 155.8 U/gds in garlic peel when substrate optimization studies were carried out by using *Aspergillus niger* (7). Similarly, in addition to garlic other nutrients like NH_4NO_3, $MnSO_4 \cdot 7H_2O$, Soya bean cake, and K_2HPO_4 showed high yield of inulinase with 76 U/gds (15). Garlic which was showing high exo-inulinase yield is used as substrate for further optimization studies.

In an attempt to optimize moisture content different percentages *i.e.* 25%, 30%, 35%, 40%, 45%, 50%, 55% and 60%. Among all moisture percentages, substrate sample from 45% showed high exo-inulinase activity (228.4±3.4) followed by 40% (198.5±5.6) and 50% (188.9.4±5.8). Fig. 2

Temperature optimization studies were carried out for the high yield production of exo-inulinase by maintaining the temperatures from 20-30°C in separate substrate bags. Substrate samples were collected at 96 h which showed high inulinase activity at 26°C (219.1±5.8). Fig. 3

Similarly, pH optimization was carried out by adjusting the pH of minimal nutrient solution. pH range maintained was 3,4,5,6,7 and 8 and the substrate samples were collected at 96 h. Among all the pH, high inulinase activity was recorded at 5.0 with an exo-inulinase activity of 213.6±4.5. Previous research reports showed 148.2 U/gds

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when garlic peels were used as substrate by using *Aspergillus niger* (7) Fig. 4.

Effect of different metal ions on the production of exo-inulinase was tested by using $MgSO_4$, $ZnCl_2$, KCl, $CaCl_2$, NaCl, $HgCl_2$ and $MnSO_4$. In the current research study, Ca^{2+} induced the exo-inulinase production with an enzyme activity of 196.3±5.6 followed by Mn^{2+} (174.4±6.2). Whereas Hg^{2+} inhibited the inulinase production with a minimum enzyme activity of 37.0±3.3.Fig 05 Research reports indicate the inducing of inulinase activity by Ca^{2+} ions by using *Aspergillus fumigatus* (16). In *Bacillus* sp. B51f, Ca^{2+} showed increase in the yield of inulinase enzyme (17).

Conclusion

From the current study, it can be concluded that *Trichoderma asprellum* RSBR08 can be produced by solid state fermentation by taking Garlic as the substrate which showed exo-inulinase activity of 199.2±6.3 U/gds. From the results of process optimization studies it can be concluded that at 45% moisture content, pH - 5.0 and temperature - 26°C, high yield of inulinase enzyme can be produced. When effect of metal ions was tested on inulinase enzyme production, Ca^{2+} induced the enzyme production whereas Hg^{2+} inhibited the inulinase production. By using the above process parameters, high inulinase production can be observed and this can be effectively used in industrial applications.

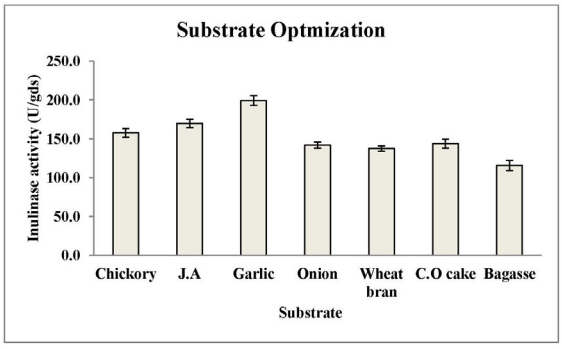

Fig. 1: Substrate optimization studies

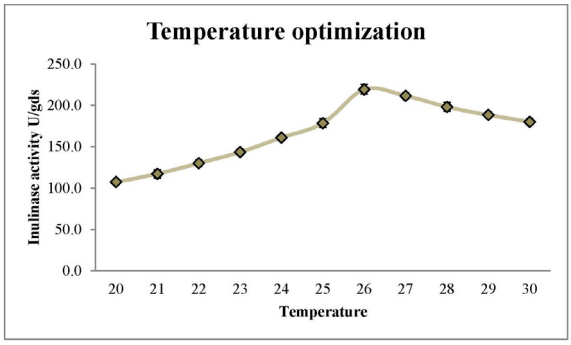

Fig. 3: Temperature optimization studies

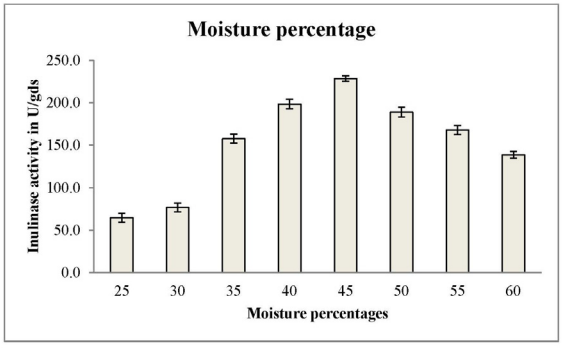

Fig. 2: Moisture percentage optimization

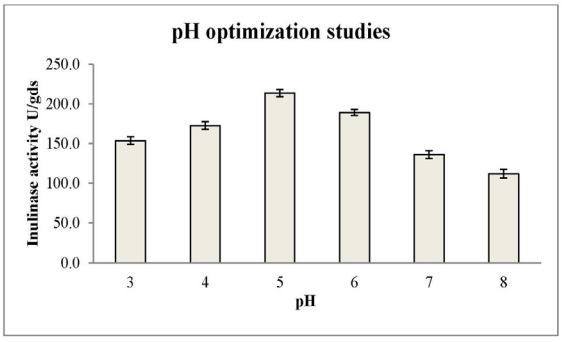

Fig. 4: pH optimization studies

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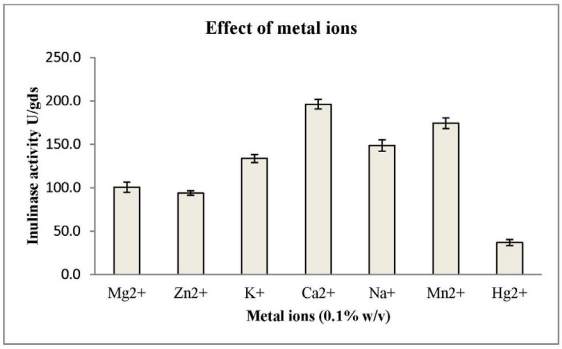

Fig. 5 Effect of metal ions on inulinase production

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Conflict of interest

Authors have no conflict of interest.

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Morphological and Anatomical Characters of *Rauwolfia serpentine* Benth. ex Kruz.

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Abstract

Rauwolfia serpentine Benth. ex Kruz. is a tropical plant of family Apocynaceae and distributed throughout the various regions of India. It is herb of medicinal importance and finds its application in Ayurveda. In the present investigation an attempt was made to study the distinguishing microcharecters of various organs of plant and histochemical localization of various substances viz., calcium oxalate, lignin, suberin, lipids etc. The salient anatomical features are useful in solving taxonomic problems and have importance in identification of crude drugs from this taxon.

Key words: *Rauwolfia serpentine*, Medicinal Plant, Anatomical characters, Pharmacognosy.

Introduction

Rauvolfia serpentina (L). Benth. ex Kurz. (Apocynaceae) commonly known as Sarpagandha is an important **medicinal plant** of Indian subcontinent and South East Asian countries

The family Apocynaceae is one of the most diversified families of flowering plants. It includes many of the most well-known tropical ornamental plants. Several plants of a family Apocynaceae are useful medicinally. *Rauwolfia serpentine* Benth. ex Kruz. found in the tropical Himalayas in lowers Hills of Himachal Pradesh, Uttaranchal, Jammu and Kashmir, and at moderate altitude in Sikkim, Uttar Pradesh, Bengal, Konkan, Assam, Burma, Sri Lanka, Andaman and Deccan Peninsula along with the Ghats of Travancore and Ceylon, Java, and Malay Penisula (Nandkarni, 2007) is a medicinal plant.

It is a small erect or suberect perennial glabrous under shrub (Plate 18.1A). Leaves are simple and undivided and are either opposite or in rings around the stem. Venation is pinnate, camptodromous and eucamptodromous. The flowers are in clusters and are often large and showy. Fruits are drupes and contains a single seed.

The plant is antihypertensive or hypotensive and sedative. It is useful in the treatment of high and low blood pressures, toxic goitres, excessive sweating, itching and in gynaecological ointments for menopause. Plant also used to promote uterine contraction during child birth (Deshpande, 2005). Extract of root is used for the treatment of diarrhoea and dysentry (Choudhury *et al.,* 1993).

Several workers have carried out investigations on *Rauwolfia serpentine* Benth. ex Kruz plant as it contains many important phytochemicals. These important compounds are alkaloids, phenols, tannins and flavonoids (Kumari, *et al*. 2013).

The present work was carried out on the anatomy of various organs *viz*., root, stem, lamina, midrib, petiole, stomata and trichomes and their significance in taxonomy and pharmacognosy.

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Materials and Methods

Stem, root, node and leaves of *Rauwolfia serpentine* Benth. ex Kruz. were collected from the field of South Gujarat region of India. Their small pieces were fixed in Formaldehyde – Alcohol – Acetic acid and preserved in 70% ethanol. The small pieces of stem, root, node and leaves where processed for microtomy. 8 – 10 micron thin transections were taken and stained with safranin O and FCF fast - green. For the stomatal studies, epidermal peels were taken with the help of forceps and stained with Delafield's hematoxylin and mounted in glycerine jelly (Berlyn and Miksche, 1976, Pal *et al*., 2013, 2015). The photographs were taken on axion scope A1 Photomicroscope of Carl Zeiss.

Results

Stem: Epidermis has rectangular cells with thin and wavy cuticle. Parenchymatous cortex lies below epidermis (Fig. 1B). Pith is parenchymatous (Fig. 1B). Many 3 - 5 celled eglandular uniseriate filiform and cylindrical trichomes are observed on the stem surface (Fig. 1C). In young stem four bicollateral vascular bundles are observed in a ring.

Vascular cambium is 2 - 4 layered. It produces more secondary xylem than secondary phloem. Xylem and phloem rays are uniseriate to biseriate. Phellogen is 1 - 2 layered (Fig. 1D). Phelloderm is parenchymatous. Phellum cells are rectangular and arranged in radial files. Some of them contain tannin (Fig. 1D). Xylem ray cells and xylem fibers have thick walls with lignin deposition. Vessel elements are solitary. Few scattered sclereids are observed in the pith (Fig. 1E). Lipid globules and starch grains are observed in pith cells of old stem (Fig. 1E, F). In old stem internal phloem is observed in the form of a complete ring below the cylinder of secondary xylem.

Root: Young root has large cortex, tetrarch xylem and pith of few parenchyma cells (Fig. 2A). Transections of old root shows a ring of vascular cambium, secondary phloem, secondary xylem and periderm. Secondary xylem is consisted of vessel elements, xylem fibers and uniseriate to biseriate xylem rays (Fig. 2B, C). Starch grains are observed in xylem ray cells (Fig. 2B). Vessel elements are present solitary or in groups of two. The phloem ray cells are uniseriate to biseriate (Fig. 2C). Their cells are short and have thinner cell wall.

Periderm is consisted of phellogen of rectangular cells, parenchymatous phelloderm and phellum of cells arranged in radial files. Large number of starch grains are present in the cells of phelloderm (Fig. 2C).

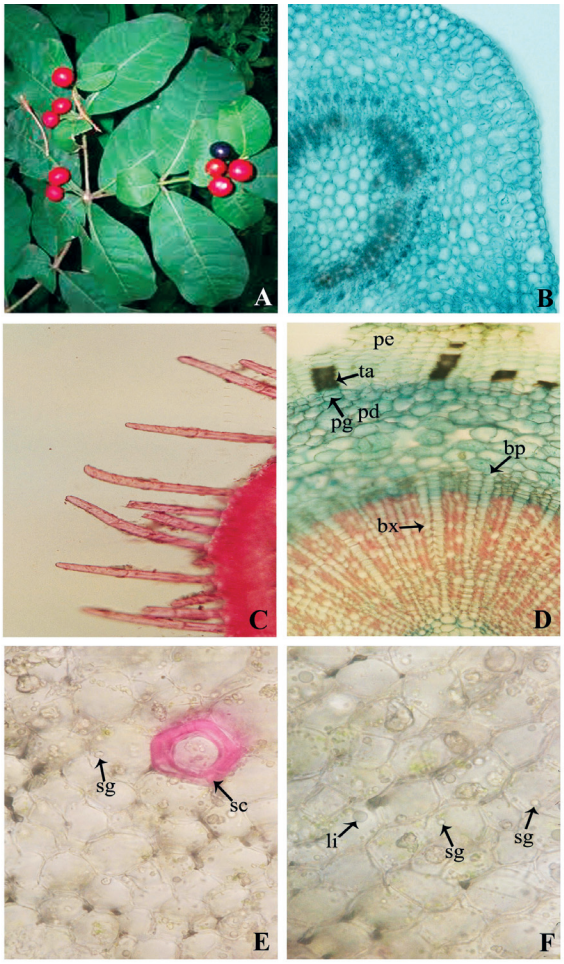

Fig. 1 A – F, A, twig of *Rauwolfia* plant. B - F, transection of stem, B - D, X480; E, F, X800. (bp - biseriate phloem rays; bx – biseriate xylem rays; li - lipid globules; pd - phelloderm; pe - phellum; pg - phellogen; sc - sclereids; sg - starch grains; ta - tannin.).

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Nodal anatomy: Node is unilacunar single trace. A curved leaf trace is supplied to a leaf (Fig. 2D).

Leaf

Lamina: Lamina is dorsiventral. Cells of adaxial epidermis are larger. Palisade cells are elongated, present in a row. Several layers of spongy cells are observed. Mesophyll strands are small and collateral.

Walls of epidermal cells is slightly sinuous (Fig. 2E). Lamina is amphistomatic. Stomata are paracytic (Fig. 2E). Haplocytic stomata with lateral subsidiary cell are rarely observed (Fig. 2E). Epidermal trichomes are present similar to stem.

Mid rib : Mid rib has a large crescentic bicollateral vascular bundle, cortex of parenchyma and hypodermis of collenchyma on adaxial and abaxial sides (Fig. 2F). Eglanudular trichomes are present similar to stem (Fig. 2F).

Petiole : Petiole exhibits similarity with midrib in the structure of hypodermis, cortex, vasculature and types of trichomes.

Discussion

The main anatomical investigations on *Rauwolfia serpentine* were carried out on leaf epidermis (Naywuame and Gill, 2008; Sharma *et al*., 1970), Wood anatomy (Lens *et al*., 2008), sclereids of the endocarp (Gupta and Lamba, 1981), underground organs (Liapunova and Gorodnianskaia, 1971; Gorodnianskaia and Liapunova, 1972), vegetative parts of *Rauwolfia* (Gorodnianskaia and Liapunova, 1973) and seedlings (Garasia, 2002).

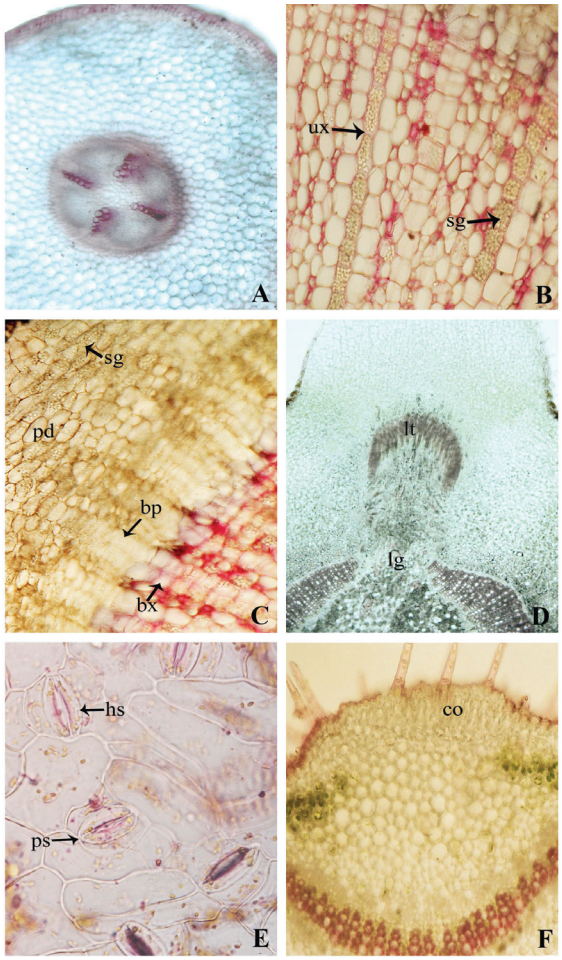

Fig. 2 A – F, transection. A - C, transection of root, A, X80; B, C, X480; D, transection of node; D, X80; E, surface view of abaxial epidermis of lamina, E, X800; F, transection of midrib, F, X480. (bp - biseriate phloem rays; bx - biseriate xylem rays; co - collenchyma; hs - haplocytic stomata; lg - leaf gap; lt - leaf trace; pd - phelloderm; ps - paracytic stomata; sg - starch grains; ux - uniseriate xylem rays.).

In the Apocynaceae the vascular system is typically consisted of bi-collateral vascular bundles at the border of pith (Metcalfe and Chalk, 1950). In *Rauwolfia serpentine* stem vascular system is formed by a ring of four separate bicollateral vascular bundles. While in the old stem vascular cambium forms a continuous cylinder of secondary xylem traverse by uni – biseriate xylem rays and internal phloem is present around the pith in form of ring. These anatomical features are remarkable characteristics of Apocynaceae (Metcalfe and Chalk, 1950; Cronquist, 1981) and found in *Rauwolfia sellowii* (Santos *et al*., 2010). The starch grains are commonly observed in parenchyma cells of cortex, pith and in vascular tissues (Esau, 1974; Cutter, 1986). In *Rauwolfia serpentine* abundant starch grains were found in parenchymatic cells of pith of stem, xylem ray cells as well as phelloderm of root. Vascular cambium forms large number of xylem fibres during secondary growth in root and stem of

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Rauwolfia serpentine. They are long with thick lignified secondary walls. They have main function of sustaining the plant, providing rigidity to the organs (Esau, 1974; Costa and Costa, 1980). According to Matcalfe and Chalk (1950) and Garasia (2002) paracytic and haplocytic stomata are common in *Rauwolfia*. Similar types of stomata are observed in the present work which differs from the report of anisocytic stomata in *Rauwolfia serpentine* leaves investigated by Panda *et al*. (2012). Panda and his co – workers (2012) also observed multicellular trichomes in *Rauwolfia serpentine* and *Rauwolfia tetraphylla*. Garasia (2002) reported various types of trichomes on leaves and cotyledons of Apocynaceae. In present study also multicellular glandular trichomes are found in the leaves of *Rauwolfia serpentine*.

Conclusion

In the present study the distinguishing micro characters of this species useful for the authentication of its organs / materials used for the preparation of drug are investigated. They are

- Stem has four bicollateral vascular bundles.
- Internal Phloem is present below xylem cylinder in old stem.
- Eglandular 3 - 5 celled cylindrical and filiform trichomes are present on stem, lamina, midrib and petiole.
- Starch grains are present in pith cells of stem; xylem ray cells of root; and phellodermal cells.
- Lipid globules are observed in pith cells of stem.
- Stomata are usually paracytic.
- Tannin is found in phellum cells of stem.
- Mid rib and petiole has a large bicollateral vascular bundles.

These anatomical characteristics which are observed in *Rauwolfia serpentine* contribute to the identification of this medicinal plant and provide valuable information that can serve as quality control parameters of the plant drug.

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Role of IoT and its Adoption in Smart Farming

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Abstract

Despite the agricultural debts and loans are waived/or written off by various Govt, the access to institutional loan are limited especially to small and marginal farmers thereby forcing them to depend on money lender and agents. The share of farm income, which made up 74 per cent of rural incomes in the 1970s, has dropped to 30 per cent in 2010. Non-farm growth has been substantial in rural India. The popular view is that non-agricultural income, which accounts for a hefty 50 per cent of rural income, is more stable (and less volatile) than agricultural income. As there is no competition in agriculture, farmers are not benefiting from agriculture and hence, forcing them to depend on non-agricultural income. The alleviation of poverty may be a true challenge while majority of the population lives in rural area with the declining share of agriculture to the GDP. NSS employment data for 2007–08 and 2009–10 show clear evidence of an accelerated shift of rural laborer to non-agricultural work and it is not an undesirable development. While private investment in irrigation and water-saving devices did increase, the largest increase was in labour-saving mechanization. Labour saving mechanization helped farmers to cope up with labour scarcity and rising wages. The agriculture sector in India suffers from poor productivity due to falling water levels, expensive credit, a distorted market, the intermediaries, controlled prices, inadequate infrastructure, and poor quality of agriculture produce compared to the international standard. Crop farming in India is labor intensive and the farming even today follows the traditional and tacit methods being used over many centuries. Agriculture has also suffered because of farmers wholly depending on the monsoon, poor irrigation facilities, use of traditional practices, farmers' poor economical status, fragmented landholdings, poor yields, lack of post-harvest infrastructure, not taking care of conservation resources and lack of farm extension. In order to mitigate the issues, ICAR has proposed in Twelfth Five Year Plan a number of new initiatives such as extramural funding for research, creation of funds for agro-innovations and agro-incubation, and setting up of an Agriculture Technology Forecast Centre (ATFC). The IoT frameworks can collect process and analyse data streams in real-time and facilitate provision of smart solutions designed to provide decision support in agriculture and help address some of the problems in agriculture. This paper briefly explains IoT principles and its architecture in agriculture, various off-the-shelf IoT applications readily available for deployment which enhances agricultural economy.

Key words: Internet of Things (IoT), Sensors, Smart farming

Introduction

Agriculture has undergone several fundamental changes during the 20th Century, including extensive dependence on farm machinery, intensive fertilizer and agrochemical management, crop breeding, high yielding hybrid varieties, and genetic manipulation. Fig 1 depicts the linkages among various stakeholders in the

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agricultural life-cycle. The extension worker is the last mile connection between farmer and various government organisations. Farmers interact with Call Centre to raise questions regarding farming and to get the responses where as markets help to purchase and sell their produce.

The adoption of Information and Communication Technology (ICT) in agriculture has played an essential role in education, training, e-services and rural development projects. ICT adoption in agriculture at village level through information centers comprising of Open Source Software has reduced the gap of digital divide in rural population especially in developing countries like India. ICT enables agricultural online surveys using mobile applications and data gathering from mobile devices to measure indicators of soil nutrient levels etc. which is analyzed using ICT tools. The delivery of ICT services is enhanced by adopting Cloud computing from anywhere, any time and any place by moving the users beyond desktop experience. The cloud based sites like Central Agricultural Portal (http://dacnet.nic.in/) in India provides a platform by offering cluster of services for all stakeholders (farmers, private sector and the Government, research scholars) to access information, avail services, collaborate and share knowledge. AGMARNET (http://www.agmarnet.nic. in) is an Agricultural Marketing Information System in India that exchanges data with market yards and provide information to all stakeholders in real time basis. A global public domain database AGRIS (International System for Agricultural Science and Technology) permits the use of keywords to perform sophisticated searches from the AGROVOC thesaurus, specific journal titles or names of countries, institutions, and authors. Cloud Computing has fundamentally changed the nature of ICT delivery over time and so also now IoT is ushering a new phase in service delivery. The combination of cloud computing and IoT can enable the resource sharing more efficiently than individually handling them. Cloud Computing, IoT and Machine Learning will play a major role and be a key part of the strategic future of ICT in the public service (3).

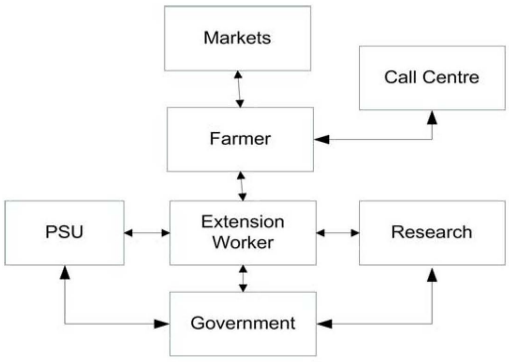

Fig. 1. Stakeholder interactions in agricultural life cycle

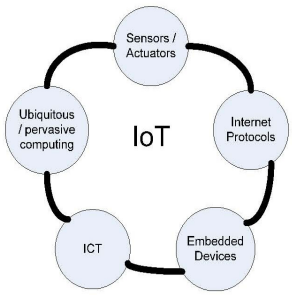

Fig. 2: IoT Eco System

Overview

Internet of Things (IoT): "Internet of Things (IoT) is an integrated part of Future Internet and could be defined as a dynamic global network infrastructure where physical and virtual 'things' have identities, physical attributes, and use intelligent interfaces. The Internet of Things is a concept in which the virtual world of information technology integrates seamlessly with the real world of things. In IoT model, things and people are connected at anytime, anyplace and by anyone, any network and any service. The Internet of Things is not the Internet of People and it is not the Intranet or Extranet of Things (4).The aim of Internet of Things (IoT) is to enable the integration and interconnection of the existing physical components, internet and the people.

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The users can interact with physical systems such as sensors, actuators etc for gathering data from these devices while communicating and interacting with other systems using the other components shown in the Fig 2. It can be seen that IoT is internet / things / semantic oriented. Internet acts as middleware between user and intelligent things in gathering data and building the knowledge base required for decision making i.e. semantic orientation of intelligent process.

Wireless Sensor Networks (WSN): The wireless sensor network consists of thousands to millions of tiny sensor nodes for sensing the environment with the limited computation and communication capabilities. The sensor nodes in the networks are battery operated with limited recharge capabilities. IoT solution interacts with the world through sensors and actuators. The information detected by sensors is measured and converted into digital data. The resolution of sensor plays an important role in measurement. The actuators are output devices like LED, display unit, motor etc. Communication between sensors to computer/Cloud is done using a number of communication protocols based on the power consumption, coverage, data rate, and cost requirement for each IoT system. The data that is collected by sensors will be transferred to servers for analysis and decision making. The controllers which talk to sensors and actuators are connected to internet either directly or through a gateway as depicted in the given below diagrams.

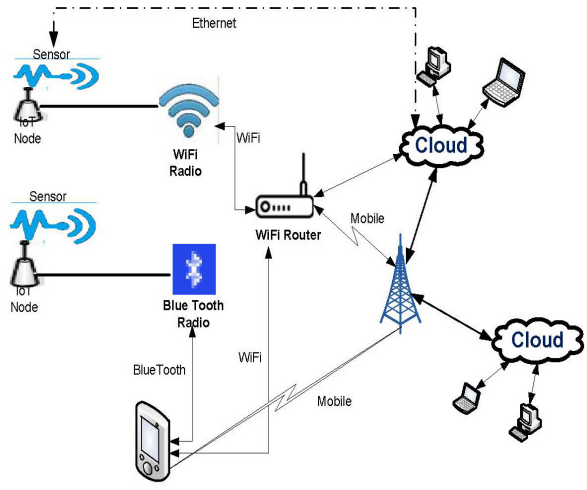

Fig. 3. IoT Network

Fig 3 illustrates how communication is sent to the cloud using wireless communication via Bluetooth, Wi-Fi and mobile. IoT devices are connected to the network through the router and have the ability to send data to the remote server on the Internet or to the Cloud services. The sensor data gathered from IoT devices are stored into the Database in the Cloud to be available for users.

The controller in the IoT node can directly be connected to cloud over Ethernet interface and can send data using HTTP protocol. If the sensor nodes implement the TCP/IP stack, then they can be considered as full-fledged elements of the Internet, which, in fact, integrates WSN with IoT. A consequence of this approach is that sensor nodes are no longer able to use specific WSN protocols and hence an IoT device acts as the web client. The data collected from sensors can be transported to the web server on the Internet and users of the net can control the attached actuators such as DC motor or light intensity. In Gateway approach, the router routes the information from one point to another. As a result, Internet hosts and sensor nodes can be able to address each other and exchange information without establishing a truly direct connection. In this approach, the WSN is still independent from the Internet, and all queries still need to traverse a gateway device. However, sensor nodes can be able to provide web service interfaces to external entities while maintaining their lower layer protocols.

Edge and Fog Computing: The data generated from billions of sensors is more challenging in terms of processing such huge data in real time. Due to latency constraints, processing such huge data from cloud is not feasible in real time. Edge

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computing paradigm has emerged solely because of real-time needs of the applications and edge devices such as routers, switches and access points play an important role in Edge computing paradigm. Edge computing pushes the intelligence, processing power and communication capabilities of an edge gateway or appliance directly into devices like programmable automation controllers (PACs). In Edge computing, the end user is closure to things / or devices than the application servers (5).

Fog computing is as a distributed computing paradigm that fundamentally extends the services provided by the cloud to the edge of the network. Fog computing essentially involves components of an application running both in the cloud as well as in edge devices between sensors and the cloud that is, in smart gateways, routers, or dedicated fog devices (6). Fog computing pushes the intelligence down to the local area network level for processing data in a fog node or IoT gateway. The chief advantages with fog computing are

1. Reduction of network traffic
2. Suitable for IoT tasks and queries
3. Low-latency requirement
4. Scalability

The vast data collected from sensors can be stored and processed either at fog node or in cloud as shown in Fig 4. At the time of design, it may be decided which part of the data stored at edge/ fog node and which part in cloud (7).

IoT Protocols: IoT can be thought of as an aggregation of different networks, including mobile networks (3G, 4G, CDMA, etc.), WLANs, WSN. Depending on the application, factors such as range, data requirements, security, speed, reliability, power demands and battery life will dictate the choice of one or some form of combination of technologies. Seamless connectivity is a key requirement for IoT. WSN is completely independent from the Internet, so it

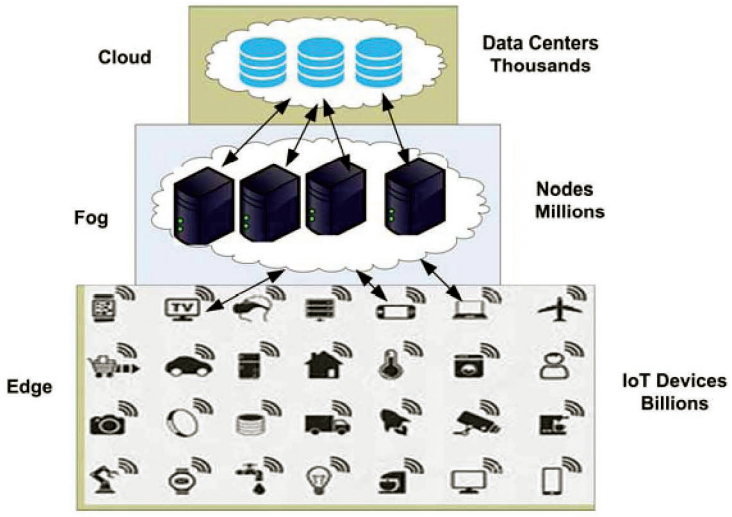

Fig. 4. Fog /Edge and Cloud network

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can implement its own set of protocols. The given below are some of the major communication technologies on offer for users and developers.

Layer	Protocols
Application	MQTT, CoAP, AMQT, HTTP, SOAP etc.
Network	6LowPAN, RPL, CORPL, IPSec, TCP/UDP, DTLS
Things / Physical and Link	802.11 WiFi, Bluetooth Low Energy (BLE), NFC (Near Field Communication), Low Power Wide Area Network (LPWAN), Zigbee, Z-Wave, LoRa and Sig Fox,

Table A – Protocols in IoT Communication

According to the Bluetooth SIG, more than 90 percent of Bluetooth-enabled Smartphone's, including iOS, Android and Windows based models, are expected to be 'Smart Ready' by 2018. Bluetooth Smart sensors are to access the Internet directly via 6LoWPAN connectivity. ZigBee is an industry-standard wireless networking technology for applications that require relatively infrequent data exchanges at low data-rates over a restricted area and within a 100m range such as in a home or building. 6LowPAN (IPv6 Low-power wireless Personal Area Network) is key IP (Internet Protocol). IPv6 is the key in enabling any embedded object or device in the world to have its own unique IP address and connect to the Internet. The longer distance communication capabilities of 3G /4G/GSM can be exploited by IoT applications. Because of cost and high power consumption in 4G, it may be used to send data over internet where the sensor data rate is low. Sigfox is designed for low data in range of 10 to 1,000 bits per second for M2M applications that run on a small battery. LoRaWAN targets wide-area network (WAN) applications with features specifically needed to support low-cost mobile secure bi-directional communication in IoT, M2M smart city and industrial applications.

IoT in Agriculture

Smart Farming : Smart farming is a capital-intensive and hi-tech system of growing food cleanly and sustainable for the masses. Smart farming based on IoT technologies will enable growers and farmers to reduce waste and enhance productivity ranging from the quantity of fertilizer utilized to the number of journeys the farm vehicles have made. In IoT-based smart farming, a system is built for monitoring the crop field with the help of sensors (light, humidity, temperature, soil moisture, etc.) and automating the irrigation system. The farmers can monitor the field conditions from anywhere. IoT-based smart farming is highly efficient when compared with the conventional approach. In IoT, sensors can be deployed across farm and farming machineries in order to enable farmers to gain an abundance of insightful data, such as the temperature of stored produce, the amount of fertilizer used, the amount of water in the soil, the number of seeds planted, storage conditions, the status of farming equipment and machinery in use, etc. Once an IoT-enabled smart system is in place, farmers can easily track a variety of environmental variables and take informed decisions.

Architecture: The key drivers (2) of agriculture for defining the architecture are given below:

1. Viability of farm enterprise and returns to investment that depend on scale, market access, prices and risk;
2. Availability and dissemination of appropriate technologies that depend on quality of research and extent of skill development;
3. Plan expenditure on agriculture and in infrastructure which together with policy must aim to improve functioning of markets and more efficient use of natural resources
4. Governance in terms of institutions that make possible better delivery of services like credit

   As per Agricultural MMP, Ministry of Agriculture, services are classified as given below.

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1. Providing information on quality pesticides
2. Providing information on quality fertilizers
3. Providing information on quality seeds
4. Providing information on soil health
5. Providing information on crop pests, insects & diseases
6. Providing information on forecasted weather
7. Providing market info on prices & arrivals of agricultural commodities
8. Providing farmer advisory services based on market information
9. Providing interaction platform & transport services
10. Providing information on Minimum Support Price & Govt. Procurement Points
11. Providing electronic certification for imports & exports
12. Providing information on marketing infrastructure &post harvest facilities
13. Providing information on storage infrastructure
14. Monitor implementation of schemes / programs
15. Providing training support to farm schools for adoption of GAPs
16. Sharing Good Agricultural Practices with farmers & trainers and providing extension support through online video

Fig 5 illustrates the layered architecture of different systems in Smart farming. The delivery channels are highlighted in the presentation layer along with the user of the system. All the agricultural services may be deployed either on fog node or in cloud based on design. The deployment of third party applications or analytics in fog or cloud is decided on the requirements of a given customer.

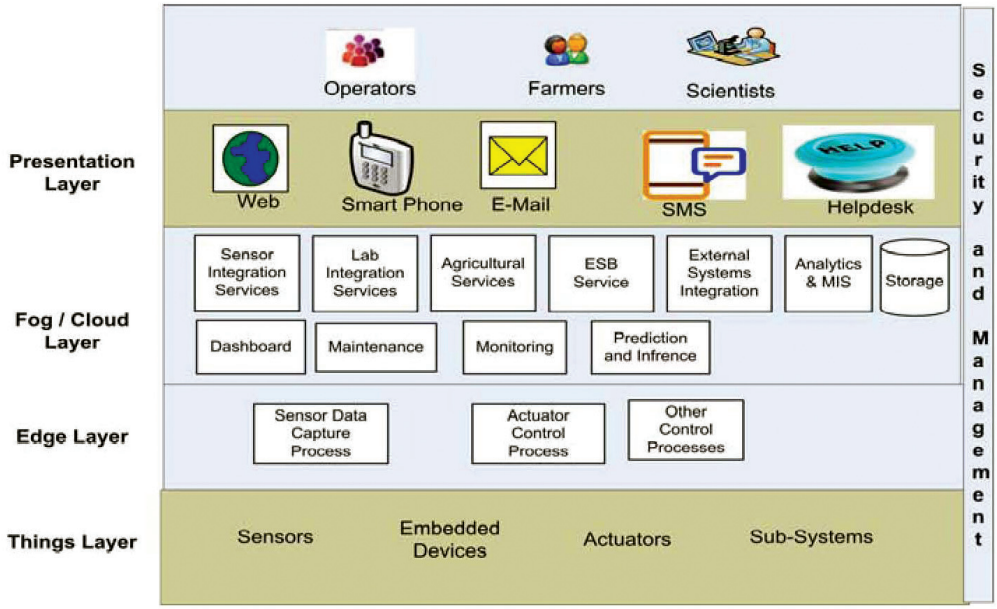

Fig. 5. Reference architecture for Smart Agriculture

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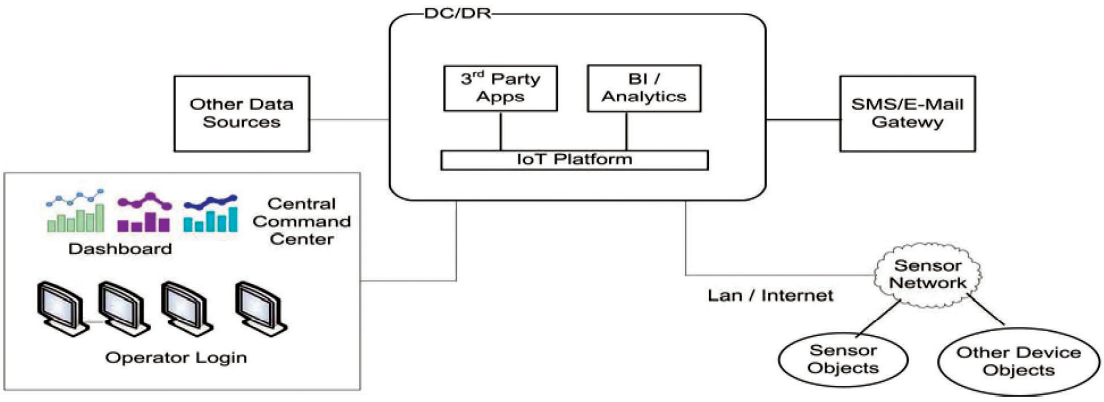

Fig. 6. Flow diagram of IoT Platform

It is clear from Fig 6, the flow of IoT interaction with different sub-systems. The integrated smart platform can integrate different IoT solutions of smart infrastructure and enables data collection and synthesis by integrating and communicating with IOT devices and third party applications by HTTP / API / or other such suitable integration methods.

Applications

The agriculture products that are offering IOT capabilities are given below.

allMETEO (https://www.allmeteo.com/)
It is a weather portal for management of your IoT based weather stations and GSM based weather stations. It offers

a. Live data access and weather station management including weather map
b. Simple user interface with live and historical data in plot and table view
c. Export to popular analytical software like MS Excel (CSV and JSON)
d. API for easy real-time data transfer into your existing infrastructure

Smart Elements (https://smartelements.io/) Smart Elements is a system of products that boost your time by eliminating the need to manually check your farms most important assets. By deploying a wide variety of sensors that report back to a very simple online dashboard and enables to make fast and informed decisions based on real time conditions

Pycno (https://www.pycno.co/) It provides the given below features:

- Aggregate your data from multiple sensors and fields
- Get notifications based on trends
- Use local weather stations and satellite data
- Predict diseases and insect growth
- Access your data securely from anywhere

Growlink (http://www.growlink.com/) Growlink Farmers are using AI to increase crop yields. Growlink Plant Health AI BETA uses Artificial Intelligence (AI), Cloud Machine Learning, Computer Vision and Sensor Data to track and predict plant health.

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GreenIQ (https://easternpeak.com/works/iot/) GreenIQ is also an interesting product that uses smart agriculture sensors. It is a smart sprinklers controller that allows you to manage your irrigation and lighting systems remotely. It's main feature are:

- Control irrigation, saves water
- Connect to leading home automation platforms
- Add accessories to your Smart Garden Hub

Crop management devices are placed in the field like weather sensors to collect data specific to crop farming in order to monitor crop growth and health of the crop.

Arable (https://arable.com/) It helps in crop management with scientific quality measurements and easy to use design.

Semios (http://www.semios.com/) Semios is the leader in onsite sensing, big data and predictive analytics solutions for perennial agricultural crops. The Semios platform is a powerful tool in yield improvement that enables growers to assess and respond to insect, disease and plant health conditions in real-time.

FarmLogs (https://farmlogs.com/) Farming software designed to execute the grain marketing decisions. It helps provide insights to improve the profitability across the entire operation. It has product suit for marketing, crop health, rainfall etc to mitigate threats and errors.

Cropio (https://about.cropio.com/#agro) Cropio is a productivity management system that facilitates remote monitoring of agricultural land and enables its users to efficiently plan and carry out agricultural operations

Sensors and Smartphone tools A number of sensing technologies and smart phone tools are used in precision agriculture, providing data that helps farmers monitor and optimize crops, as well as adapt to changing environmental factors including (8):

S.No	Sensor Type	Description	Smartphone Tool Name	Description
1	Location Sensors	determine latitude, longitude, and altitude to within feet	Camera	Provides pictures of leaf health, lighting brightness, chlorophyll measurement, and ripeness level.
2	Optical Sensors	use light to measure soil properties	GPS	Provides location for crop mapping, disease/pest location alerts, solar radiation predictions, and fertilizing.
3	Electrochemical Sensors	provide key information on pH and soil nutrient levels	Accelerometer	Helps determine Leaf Angle Index.
4	Mechanical Sensors	measure soil compaction or "mechanical resistance	Gyroscope	Detects equipment rollover.
5	Dielectric Soil Moisture Sensors	assess moisture levels in the soil		
6	Airflow Sensors	measure soil air permeability		
7	Agricultural Weather Stations	Combination of sensors provide information such as air temperature, soil temperature rainfall, leaf wetness, chlorophyll, wind speed, dew point temperature, wind direction, relative humidity, solar radiation, and atmospheric pressure.		

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Conclusion

Agriculture has become a non-profitable and risky profession. Agriculture in India has not benefited from the advancement in technologies such as machine learning, IoT and cloud computing despite depleting resources such as low land size, high labor costs and uncertainty factors such as weather , market prices etc. Adoption of latest technologies in agriculture is a necessity for Indian farmer to stand up to the international trends. Machine learning algorithms such as classification, SVM, regression etc. are widely being used in agriculture to predict the crop yield, forecasting weather and crop decease prediction etc. The convergence of IoT and cloud enables connection of devices through internet and facilitates monitoring activities such as irrigation, forecasting, remote monitoring of crop and soil health and access of information to users which helps accomplish savings by reducing costs. Smart farming or smart agriculture solution offer benefits in terms of lower cost, enhanced quality of production, remote monitoring and equipment monitoring etc. (9). The Govt. of AP in partnership with the Bill & Melinda Gates Foundation actively promoting smart farming and development of technical solutions that solve agricultural challenges faced by small and marginal farmers. The vendors have demonstrated to Govt. AP the technical solutions using GIS Geographical Information System), Remote Sensing, LiDAR, Drone, RFID (Radio Frequency Identification), GPS (Global Positioning System) and High- Precision Ground Surveying (DGPS, Total Station, GPS) Technology, supply-chain for agri stakeholder making use of IoT and machine learning algorithms for data analysis (10).

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Cystic Fibrosis in Human - A review

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Abstract

Cystic fibrosis is an inherited Autosomal recessive monogenic genetic disease in a various human population worldwide. The disease, characterized by the accumulation of thick and sticky mucus that can damage many body organs starting with respiratory system associated with chronic digestive system most severely affected leading to death in 90% of patients. Various mutations in the *CFTR* gene located on human chromosome 7 with specific location 7q31.2 are the cause of disease. The *CFTR* gene synthesizes a protein called the cystic fibrosis transmembrane conductance regulator that controls the movement of salt and water in and out of human body's cells. Therefore, *CFTR* is an ion channel protein that transports chloride ions across the membranes of cells that line airways, glands, and the digestive tract. Chloride ions balance the water, making mucus thick or thin. The article discusses background, prevalence, etiology, complications, diagnosis, possible treatments and future development in clinical research of cystic fibrosis.

Key words: *CFTR* gene, Cystic fibrosis, mutation, nonsense mutation,

Introduction

Though the disease was not named in the history of medical science, but people were aware for cystic fibrosis since 1857. It was popularized by German saying 'the child will soon die whose brow tastes salty when kissed' (1). The clinical entity of disease was first described by Dorothy Andersen in 1938 (2) and it was considered as a lethal disease of babyhood. Genetic cause and inheritance pattern of the disease were described in 1946. During 1950s the sweat test was developed as a result of discoveries made by Paul di Sant'Agnese during the heat wave in New York in 1953 (3). It was later standardized by Gibson and Cooke in 1955 (4). In 1955, Cystic Fibrosis Foundation was established in the US and chloride transport was identified as the basic physiologic defect of CF in 1983. In 1985, gene causing cystic fibrosis was narrowed down to chromosome number 7. Finally, Professor Lap-Chi Tsui and his colleagues identified the specific fault in cystic fibrosis transmembrane conductance regulator (*CFTR*) gene in 1989 (5).

Cystic fibrosis is an inherited Autosomal recessive monogenic genetic disease in humans. The disease, characterized by the accumulation of thick and sticky mucus that can damage many body organs starting with respiratory system associated with chronic digestive system most severely affected, leading to death in 90% of patients (6). Mucus is a substance that protects the linings of the respiratory system, digestive system, reproductive system, and other organs and tissues. Mucus is a viscous fluid containing inorganic salts, glycoproteins, antimicrobial enzymes, immunoglobulins and water and produced from cells found in mucous glands. In cystic fibrosis, the body produces mucus, which is abnormally thick and sticky. This abnormal

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mucus can block the windpipe of the respiratory system and can lead to severe breathing problems and bacterial infections in the lungs. Over time, mucus and infections result in permanent lung damage, including formation fibrosis and cysts in the lungs. Cystic fibrosis is a fatal disorder of childhood. Whereas, with improved treatments and better management many people with cystic fibrosis now live well into adulthood. The Cystic Fibrosis Foundation (CFF) is projecting a life expectancy of 37 years for CF patients currently (7). Whereas, a UK study predicts that a CF patient can expect to live more than 50 years of age (8). In countries with limited resources like India, the survival of children with CF is lagging behind considerably as compare to the developed countries.

Genetic inheritance: The CF is monogenic autosomal recessive genetic disease that means when a mother carrier of CF mates with normal father will produce 50% carrier and 50% normal progeny (Figure 1). Similarly, when both father and mother are carriers, they will produce 25% normal, 50% carrier and 25% affected for CF (Figure 2).

Symptoms: The prominent sign of the CF in affected babies as they have salty skin that can be realized when mother or any person kisses baby. .The disease appears in many organs, but mainly upper and lower respiratory tracks, pancreas, digestive system, and reproductive tracts (9). For most patients, lung disease is the serious problem that sometimes causes death of a person. Respiratory system complications include bronchiectasis, pneumonia, nasal polyps, hemoptysis, pneumothorax and eventually respiratory failure (10). An affected person suffers from persistent cough with phlegm. Wheezing or dyspnea (shortness of breath) on exertion is commonly observed in patients of CF.

Digestive system complications elucidate nutritional deficiency. Affected babies have a blockage intestine that occurs shortly after birth. Nearly 20% of people with cystic fibrosis develop diabetes by age of 30. The pancreas is the vital gland responsible for the digestion of carbohydrate, protein and lipid through the secretion of various digestive enzymes into the duodenum (11). The concentrated mucus secretion causes obstruction of the ducts inhibiting secretion of digestive enzymes. CF condition is associated with abdomen pain, diarrhoea, heartburn, severe constipation, common gastrointestinal problems, etc. Other complications cause poor digestion and absorption due to small intestine bacterial overgrowth, enteric circular muscle dysfunction, abnormal intestinal mucus, and intestinal inflammation. As a result, frequent greasy and bulky stools with difficult bowel movements are also observed in the CF patients. Because of poor digestion and absorption, patients exhibit poor growth and less weight gain sometimes in spite of a good appetite and food intake.

The majority of adult males with CF (99%) is characterized by congenital bilateral absence of vas deferens (CBAVD) or blocked vas deferens, which carry sperm. CBAVD is encountered in 1-2% of infertile males without CF (12). Females with CF are found to be less fertile than normal healthy women. In females with CF, delayed puberty and amenorrhoea are common due to malnutrition. CF in females is associated with congenital absence of the uterus and vagina (13). Epididymal obstruction (14), bilateral ejaculation duct obstruction with seminal vesicle abnormality (15) was also reported in CF patients.

Other complications may include Osteoporosis, Electrolyte imbalances and dehydration manifesting as increased heart rate, fatigue, weakness and low blood pressure, etc.

Occurrence: There are approximately 70,000 worldwide cases and probably 1000 new cases are added every year. CF is very common in the white population of northern European ancestry having 1 in 2000–3000 births as reported by the Cystic Fibrosis Foundation (16, 17), and less in Asian- Americans having 1:30,000 newborns (18). As reported by the National Institute of Health,

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USA, (19), the Cystic fibrosis (CF) is the most common life-shortening disease among the white population of the United States, affects more than 30,000 people in the United States and 80,000 people worldwide. The incidence varies considerably among different ethnic groups and country. The birth prevalence is estimated to be approximately 1 in 2500 children born in the United Kingdom (20). It is less common in African Americans (1 in 15000), American black population (1:17,000) and the Native American population (1:80,000) (18, 21). The total births in India during year 2012 were estimated to be 27.271 million (22). The number of children born each year with CF may be approximately 10908 presuming incidence to be about 1 in 2500 live births (5). However, most of these children are dying may be due to poor health condition, malnutrition, ignorance for diagnosis and non-availability of diagnostic tests.

Genetic cause: Cystic fibrosis transmembrane conductance regulator (*CFTR*) gene located on human chromosome 7 with specific location 7q31.2. Normally, the *CFTR* gene provides instructions for making a protein called the cystic fibrosis transmembrane conductance regulator. This protein controls the movement of salt and water in and out of human body's cells. Therefore, CFTR is an ion channel protein that transports chloride ions across the membranes of cells that line airways, glands, and the digestive tract. Chloride ions balance the water, making mucus thick or thin (Figure 3). When Chloride ions do not pass through due to absence of CFTR membrane protein, the mucus viscosity becomes thick, which cannot be moved by the cilia of epithelium cells. The thick mucus attracts invading pathogens which becomes a layer over mucus and develops infection.

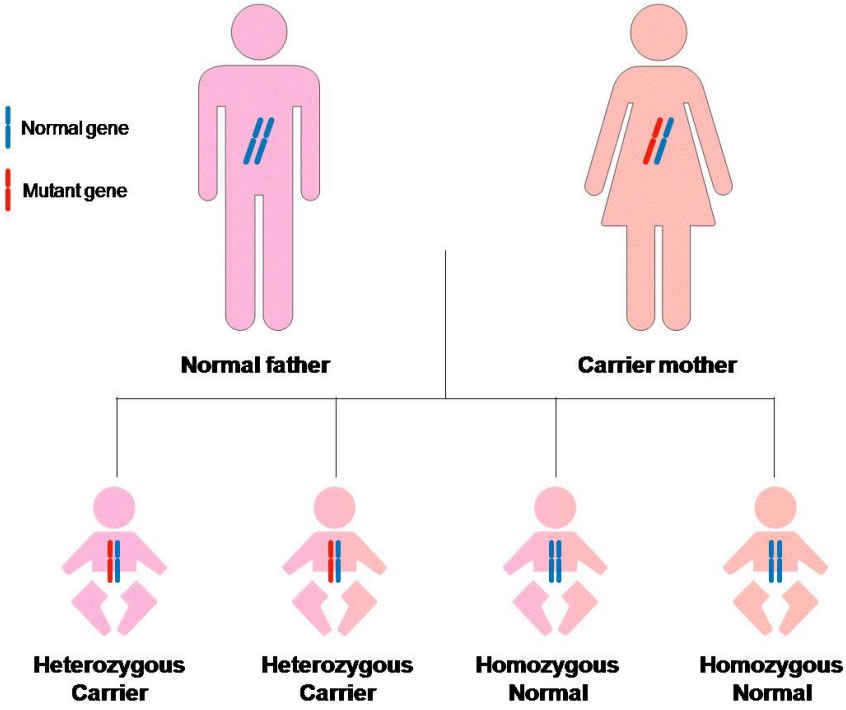

Fig. 1. Heterozygous carrier and homozygous normal

Cystic fibrosis in human

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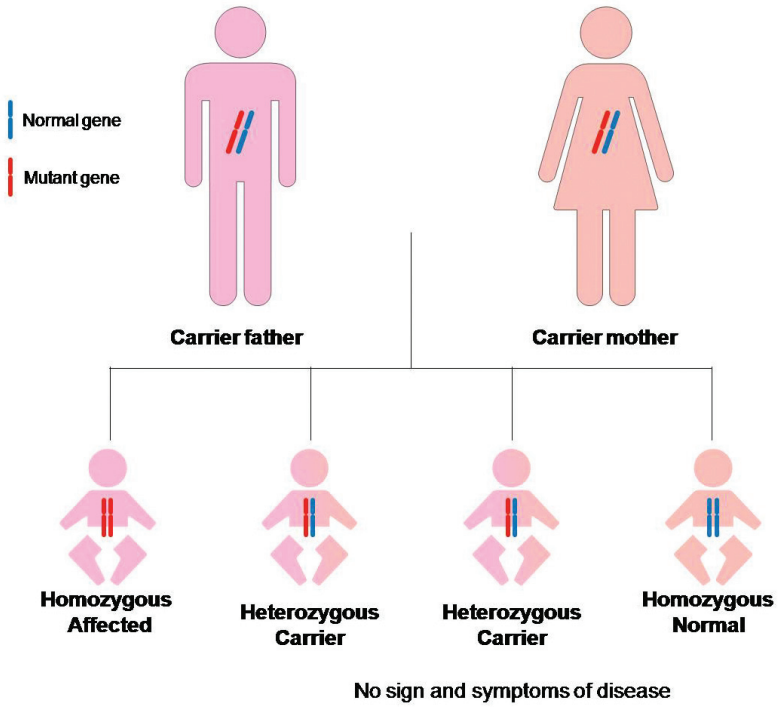

Fig. 2. Affected, carrier and normal

Because of the bacterial infection, massive neutrophil infiltration takes place that releases elastase (proteases enzyme). The protease enzymes counteract with the lung antiproteases resulting in tissue destruction (23). Additionally, degranulating neutrophils release large quantities of nucleic acids and cytosol matrix proteins contributing to the mucus hyper-viscosity (24) that causes a narrow passage of the airways, glands, digestive system, etc. When it happens in exocrine gland like pancreas it develops fibrosis and cyst. *CFTR* is highly expressed in the pancreas, particularly in the small intercalated ducts that connect the acini (25). Deficiency of functional CFTR in CF thus leads to decreased ductal cell secretions of chloride ions (Cl"), water and bicarbonate (HCO3"), which also reduces the pH (26, 27).

More than 2,000 mutations in the *CFTR* gene have been identified in people with cystic fibrosis. Most of these mutations change amino acids in the CFTR protein or delete a small amount of DNA from the *CFTR* gene. The most common mutation, called delta F508, is a deletion of one amino acid (phenylalanine in the tenth exon) at position 508 in the CFTR protein causing frame shifting. The delta F508 (ÄF508) is also renamed as p.F508del, constitutes about 70% of the total cases worldwide. As a result of ÄF508 mutation in the *CFTR* gene, it produces abnormal channel of CFTR protein, which breaks down shortly after it is synthesized, so it never reaches the cell membrane to transport chloride ions. The mucus becomes thick because of less water and no ions transportation in and out of the cell membrane. The normal allelic variant of this gene is about 250,000 base pairs long with 27 exons. The product of this gene, CFTR protein, is made of 1480 amino acids and is a member of the ATP Binding Cassette (ABC) transporter super family (28).

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Since AF508 was reported, a number of other, different mutations have been described in the CFTR locus. Granell et al. (29) reported 84-bp deletion in exon 13 of the CFTR gene in a six month old female baby with signs of CF. the 84bp deletion was detected by DNA amplification and direct sequencing of 500 bp of the 5' end of exon 13 and, as a consequence, 28 amino acid residues should be lost in the regulatory domain of the CFTR protein.

The DNA samples from 16 Hutterite CF families were analysed. The Hutterite most inbred population in North America. A new CF homozygous mutation, M1l01K was identified in exon 17b by Polymerase Chain Reaction- single -strand conformation polymorphism (PCR-SSCP) analysis of a 263bp segment of amplified DNA. Sequencing analysis identified the mutation as a T-to-A transaction at position 3434, leading to a predicted change of methionine (codon 1101, ATG) to lysine (codon AAG). The mutation was associated with maldigestion in three siblings (30). Another mutation, M1101R, which is a transversion of T to G at the same nucleotide position and should also result in a basic amino acid substitution, were identified by other investigators in a Turkish CF patient who was AF508/M1101R and had pancreatic insufficiency (W. Lissens et al - personal communication).

Severe pancreatic disease, but the mild pulmonary disease with nonsense mutations in each CF gene was observed in one of African American patients (31). The genetic investigation revealed that a patient with two nonsense mutations (stop codon); R553X and W1316X in the CF gene had exhibited an intense degree of reduced quantity of CFTR mRNA in respiratory epithelial cells. The nonsense mutation R553X (C'!T at nucleotide 1789) occurs in exon 11 of the CFTR whereas, W1316X (A'!G at nucleotide 4079)1 occurs exon 21 gene and produces a truncated protein missing the regulatory domain, leading to a reduction or absence of cytoplasmic CFTR mRNA (32). However, when mRNA is severely decreased or undetectable, the respective protein product is absent. Occasionally, nonsense mutations are associated with normal mRNA levels, but truncated proteins (33). Hamosh and his group reported a compound heterozygote for this R553X

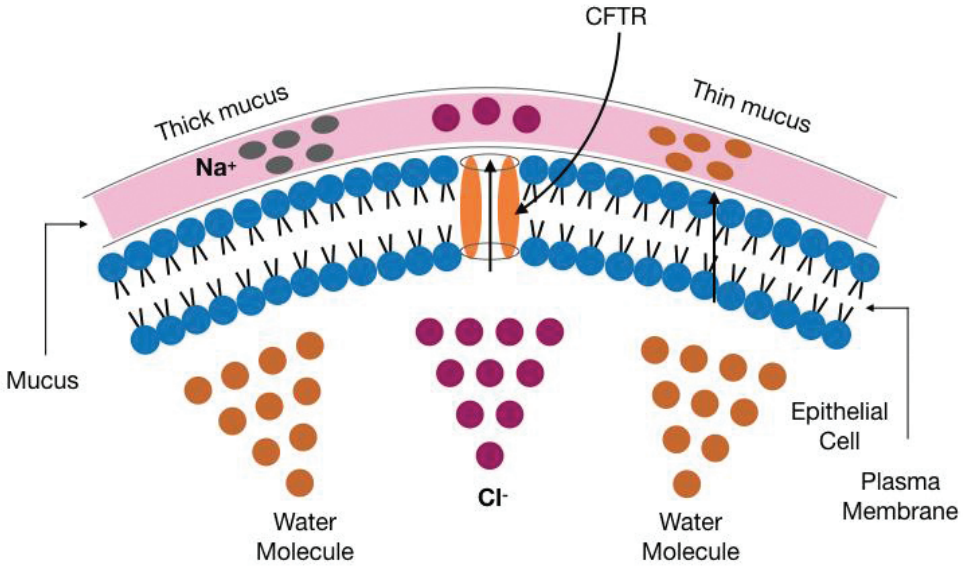

Fig. 3. Ion exchange through CFTR protein resulting thin and thick mucus

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mutation and the S549N missense mutation in another patient.

In the UK, a new mutation G551D (G'!A at neucleotide 1784) in exon 11 was reported along with Delta F508 and R553X mutations in 111 children and teenagers with cystic fibrosis (34). A nonsense R1162X mutation (C'!T at nucleotide 3616) in exon 19 in nine CF patients characterized by mild pulmonary disease phenotypes was reported (35). A new mutation G542X (G'!T at nucleotide 1756) in exon 11 of the CFTR gene that was responsible for a stop mutation in codon 542 was found in Canadian patients (36). The same mutation was observed in a Belgian patient. The G542X mutation accounted for 7.3% of the CF chromosomes in Belgium, being probably the second most frequent mutation (37). A novel mutation *1429 del7bp* was also reported in a one year old Hispanic female in combination with the well-established G542X mutation in exon 11 (38). Currently, there are 2089 mutations listed in the CFTR mutation database includes missense, Frameshift, Splicing, nonsense, deletions, etc. and most of them are very rare.

Diagnosis of CF: The primary diagnostic test for cystic fibrosis is the measurement of sweat electrolyte levels (39). Patients with the disease reveals the greater chloride value (>60 mmol/L) than normal and baby skin tastes salty when kisses. The UK now has a screening programme for all newborns for cystic fibrosis using the Guthrie blood spot test (40) and positive samples are then tested for the CFRT gene mutation. A blood test of a baby to estimate the levels of a chemical produced by the pancreas called immunoreactive trypsinogen (IRT) is also a very useful test for diagnosis of CF.

However, CF results in thick and sticky mucus obstructing the pathways leading to serious lung infections especially pseudomonas. Lung infection may be diagnosed by simple chest X-rays indicate inflated lungs, fibrosis in lung and scaring. A sinus X-ray shows the signs of sinusitis. Similarly lung function test, sputum culture can also help in the diagnosis. If CF is confirmed, pulmonary radiographs are used to monitor disease progression. Radiographs also are commonly used as a diagnostic tool for patients with symptoms reflective of CF that have not been previously diagnosed (17).

Genetic test is the appropriate to detect mutations in the *CFTR* gene. As mutations are large in number in the CFTR gene, it is advisable for PCR and DNA sequencing, and full mutation scan of the gene. The screening test for people without a family history of CF will also be done on the most common gene mutations, and so cannot be said to be 100% accurate. The most common mutation is delta F508 (ÄF508) in 10th exon exists in 70% cases that can be carried out by simply PCR-sequencing. When this mutation is not the cause of CF, one has to find out other mutations in *CFRT* gene preferably by the Ambry Test. The test is a full mutation scan of the CFTR gene by temporal temperature gradient electrophoresis analysis (TTGE) followed by dye terminator DNA sequencing of suspect regions. The Ambry Test or exome sequencing covers all *CFTR* exons and at least 20 bases 5' and 3' into each intervening sequence, and select deep intronic mutations (38).

Treatment and management: Presently, there is no cure for cystic fibrosis but treatments are available to manage the symptoms, prevent complications, and make the condition easier for the patients to live with.

To prevent and control lung infections of CF patients, wide range of antibiotics; azithromycin, tobramycin, aztreonam and levofloxacin, ciprofloxacin, cephalexin, amoxicillin and doxycycline, depending on the severity are administrated. These medicines also include inhaled forms to control respiratory inflammation (41, 42). In order to reduce viscous, thick and sticky mucus from the lungs and to dilate the airways, bronchodilators like beta-agonists are advised to inhale with humidify oxygen therapy. Inhalation of Dornase Alfa (synthetic protein or pulmozyme) can also be useful that breaks down

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excess DNA in the pulmonary secretions of people with cystic fibrosis and reduce the risk of respiratory tract infections (43,44). A lung transplant may be required if the lungs are damaged.

For gastrointestinal track blockage in CF patients, oral rehydration therapy (ORT) to replace fluid to prevent and treat dehydration is usually practiced. Osmotic laxatives (stool softener) are useful and hyperosmolar contrast enemas are given in case of distal intestinal obstruction syndrome (DIOS). Electrolyte intestinal lavage solution (washing solution) is also very effective agent in the treatment of chronic constipation in the CF patients (45). Medicines are given to patients so that they can absorb food better for digestion and special diet and food supplements are provided to prevent malnutrition. Pancreatic insufficiency may be overcome in CF patients by the pancreatic enzyme replacement therapy (PERT) (46). PERT is the use of medications that contain enzymes which are produced by a normal pancreas. These medications contain proteases to digest protein, amylases to digest carbohydrates and lipases to digest fat. Digestion of protein, carbohydrates and fats helps prevent malabsorption. Providing appropriate nutrition and preventing dehydration, a high calorie fat diet, A D E K vitamins and minerals are supplemented in CF patients. Additionally, sodium chloride is also supplemented to the patients depending on age and environmental conditions (47).

Development approach in treatment: The current and future therapeutic targets are mainly focused on correcting structural and functional abnormalities of the CFTR protein. These therapies include messenger RNA therapy, DNA or gene therapy, and gene editing. All of these therapies can be possible in the early stages of development so that normal and functional CFTR protein may be synthesized. CFTR modulators like *I vacaftor* is a drug approved by FDA used to treat cystic fibrosis in people with certain mutations in the CFTR gene (primarily the G551D mutation), which account for 4-5% cases of cystic fibrosis (48). However, it is not found to be effective in the most common mutation F508del. Another CFTR modulator, lumacaftor has shown favourable results in F508del mutation common in 72% patients (49). Most promising current drug Ataluren has shown to improve chloride transport in CF patients with nonsense mutation (50). Limitations are also observed in using CFTR modulators which includes some time non-significant effects, other daily symptomatic treatment, side effect, cost effective treatment and no guarantee of cure. However, DNA screening of heterozygosity or suspected parents and prenatal molecular screening can reduce the bourdon of CF patients in the population.

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Obesity, Cancer and Cachexia

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Abstract

Obesity is a modern times malady of globalized world and it is linked to various types cancer. The cachexia is a multifactorial paraneoplastic disease and it is also associated with obesity and cancer. The main features of cachexia are loss of muscle mass, fat mass, nausea, vomiting, fatigue, increased systemic inflammation and associated involuntary weight loss. This may be described as a result of destructive communication between tumor cells, adipose tissue, skeletal muscle characterized by an inflammatory response by the host against tumor cells and tissues. Due to all these cellular and molecular events, systemic inflammation is considered as a major feature in cachexia progression and maintenance. There are many other primary, secondary and tertiary molecular events and mechanisms are associated with these diseases. In this mini review, we briefly describe the inter relation between obesity, cancer development and cachexia and their therapeutic interventional approaches.

Key words: Obesity, Cachexia, adipose tissue, inflammation.

Introduction

Obesity is a major leading global health problem of 21st century (1). It is also a major causing factor for various diseases, very importantly it is associated with development and metastasis of almost all types of cancer (2). Cancer cells can easily access and get large amount of energy and nutrition from neighboring adipose tissue via blood vessels and small capillaries (3). Obese patients have high risk and more chances of tumor growth, development and metastasis (4). Dysregulated cancer cells release various cellular and molecular factors and these factors will stimulate adipocytes of adiposetissue (5). This will lead to release large amount of energy from stimulated adipocytes in the form of release of fatty acids and glycerol. Unregulated stimulation aids in further progression of tumor growth (3, 4, 5). This will also lead to migration of tumor cells from one organ to other distant organ via blood vessels and this process is called tumor angiogenesis (6). They start growing in newly lodged remote area of the body other than their site of origin. Again, they will start growing bigger and form bigger tumor in new place and this property of cancer cells is better known as tumor metastasis (7). In other words, due to increased tumor burdencancer cells requireeven larger amount energy and nutrition (8). Increased cellular communication between tumor cells and adipose tissuewill results in rapid and continuous lipolysis. Adipocytes willlose all the stored lipids, fats and energy (9). These starved adipose tissues will start losing various structural and functional proteins inside their cells (10). At this stage of the cancer, tumors cells will starts getting energy from other source especially from skeletal muscles of that cancer patient. Finally, even skeletal myocytes will also start undergoing proteolysis resulting in complete loss of both adipose tissue and skeletal muscle (11). This complete loss of adipose tissue

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and skeletal muscle in cancer patient is called cancer cachexia (10,11). Therefore, these cancer cells depend on adipose tissue and skeletal muscle for their growth and nutrition and utilization of complete energy from fat mass and muscle mass leads to cancer cachexia.

The causes and molecular mechanism of cancer cachexia : The exact cause and its molecular mechanism involved in cancer cachexia is still elusive. Available published data from various studies clearly shows that it is a multifactorial disease (12). Primary symptoms of this disease are loss of appetite, body weight, increased energy expenditure and metabolic activity, anorexia with anemia, finally leading to asthenia, characterizes the morbidity status of cancer cachexia (13). Due all these deleterious effects this will proceeds to respiratory failure and myocardial infarction (14).

Adipose tissue breakdown: Adipose tissue breakdown is one of the very important features of cachexia (15). In obesity accumulation of fats and lipids takes place in adipose tissue. In cachectic patients loss of adipose tissue has seen and is primarily due to an increase in lipolytic activity by different enzymes (16). Due to this increased lipolytic activity these cachectic patients immediately lose their adipose tissue mass and shows an increased turnover of glycerol and freefattyacids in blood streamin comparison with cancer patients with no weight loss or healthy subjects with normal weight (17). There are various molecules are involved in adipose tissue breakdown. One of the very important factors involved in cancer cachexia islipid mobilising factor (LMF), which is atumour-secretedcatabolic factor with molecular weight of 43 kDa glycoprotein and it acts directly on the adipose tissue and stimulates the release of free fatty acidsandglycerol (18).

Inflammation: Obesity, cancer, cachexia and inflammation are interdependent relative terms (19). Inflammation plays a major role in all these diseases. Human cachexia shows elevated levels of various inflammatory cytokines such as TNF-α, Interleukin-1, Interleukin-6, IFN-β etc (20). These inflammatory cytokines are produced by cancer cells, adipocytes and skeletal muscle. Therefore, cachectic patients may have deleterious effects from all these cells during the disease progression (21).

Muscle metabolism: Large amount of skeletal muscle loss is one of the very important symptoms of cachectic patients (22). There are numerous cellular signaling pathways plays a major role in activating muscle loss and catabolic activity, this will lead to an increase in whole body protein turnover and protein breakdown (23). Protein catabolic pathways are also associated with muscle loss[24]. Here, both proteasome mediated protein degradation and lysosome meditated protein degradation plays a major role (24). But, due the dysregulation in protein synthetic pathways, synthesis of new functional and structural proteins may not take place in these cachectic patients.

Oxidative stress: Almost all cancer patients at very advanced stage of disease progression shows pathological symptoms such as loss of appetite, nausea and vomiting (25). Due to these symptom cancer patients may lack the supply of very essential nutrients such as glucose, proteins, lipids, vitamins and micro elements. This will lead to the malnutrition state and imbalance in the availability of free radicals and antioxidants (Vitamin A, Vitamin C,Vitamin E, beta-carotene, lycopene, lutein, selenium, manganese)in cachectic cancer patientsbody (26). This will increase the number of free radicalsor free oxygen-containing molecules with an uneven number of electrons in these patients and this will eventually be leading to the accumulation of Reactive oxygen species (ROS) (27). This will again make them easily react with chemicals in the body and cause large number of biochemical reactions and chain of harmful effects. Natural antioxidants which are ingested through the food molecules that can donate electron to a free radical without making themselves unstable (28).

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This will stabilize the free radical and makes them to become less reactive molecules. It is a fact that cachectic cancer patients lack these antioxidants and stabilizing mechanism, thereforeaccumulation of large number of free radicals creating more worsen scenario.

Different stages of Cachexia

Pre-cachexia: Pre-cachexia is an early stage condition of the diseaseand it starts with loss of appetite and thedysregulation is various metabolic activitiesand physical changes with substantial involuntary weight loss (d" 5%).

Cachexia: Cachexia is a middle stage. In this stage, patients have alreadyloss of >5% of their total body weight along with additional loss of 2% body weight and <20 kg/m2 loss of body mass index.

Refractory cachexia: Refractory cachexia is a final stage. In this stage it's very difficult to treat patients, they are not responsive to any treatment options. Symptoms are characterized by low performance status andtheir external appearance looks like almost a walking skeleton and they may have maximum expected survival time of less than 3 months (29).

Prevention and treatment options for cachexia

Nutrition: The role of proper diet and nutritional interventions is very much required, and it is an essential part of prevention and treatment option for cachexia (30).It is very difficult task to treat cachexia by single drug or therapy (31). It can be prevented and treated using a multimodal and multidimensional approach (32). Because, cachexia itself is a pathological condition in which lack of an adequate energy and nutrient supply is exist and malnutrition condition is primary triggering factor for muscle and fat mass loss.Therefore, in this condition muscle mass and fat mass cannot be reversed or increased or stabilized by sudden over or hyper nutrition. Cachexia patients shows symptoms of loss of appetite and reduction in food intake and this can be reversed slowly with beneficial nutrition (31).

Good nutritional supplements with all the essential micro, macro nutrients and carbohydrates, proteins, lipids, vitamins can be provided to cachexia patients and supplementation of these diet should be started very early stage of that patients rather than delayed until at end stage where there is an advanced degree of body weight loss (33,34).Sometime proper diet can become lifesaving elixir for these patients (35). In many cases, these efforts may fail because cachexia is not a simple malnutrition or starvation, it is also associated with tumor cell mediated inflammation and also skeletal muscle and adipose tissue may have lost their biosynthetic capacity along with their mass. Therefore, metabolic modulation of adipose tissue and skeletal muscle function is very much essential (36).

Along with just basic nutrition (carbohydrates, proteins, lipids and vitamins)the nutritional intervention using nutraceuticals, dietary compounds such as Withaferin A, Curcumin, Resveratrol, Quercetin is need of the time to counteract inflammation, proteolysis, apoptosis, necrosis, autophagyand other molecular mechanisms involved in the pathogenesis of cancer cachexia is required (37).

Pharmacologic modulators: Synthetic or natural pharmacological modulators have played a beneficial role in cancer cachectic patients wellness and recovery. These modulators of pharmacologic therapies for cancer cachexia have mainly focused onadipose tissue and skeletal muscle (38). They have been used to improve the appetite, modulating deleterious inflammatory effect and interfering with anabolic and catabolic metabolic pathways which are involved in adipogenesis, skeletal muscle activity (39).

Cachexia is a very complex disease with various mediators and regulators (40). It cannot be cured by a single pharmacological modulator or a method. There are many modulators are available in the market and many modulators are continuously emerging at pre-clinical and clinical experimental level. Therefore, various scientists

and clinicians are trying to include combinatorial integrated approach to treat this disease (38).

Therefore, these pharmacological agents or modulators for the treatment of cancer cachexia can be classified into 4 groups based on their mode of action. They are as follows :(1) Appetite stimulants, (2) Cytokine modulators, (3) Anabolic agents and (4) Combination therapies.

Appetite Stimulants: There are several classes of pharmacological modulators have been studied and used as stimulants of appetite. So that to make cachexia patients canshowmore desire for food and eat more food. The best-established appetite modulators include various hormones such progestogens, megestrol, medroxy progesterone acetate, corticosteroids, ghrelin, circadian rhythm associated melatonin, erythropoietin, metoclopramide, dronabinol, fish oil, interferon, non-steroidal anti-inflammatories, nandrolone, antidepressants such as mirtazepine, atypical antipsychotics such as olanzapine, Anamorelin, Cannabis Sativa, Nabilone and many more (41).

Cytokine modulators: The cytokines are a broad category of small proteins with molecular weight ranges from ~5-20 kDa that are important in cell-cell communications and cell signaling. These small proteins play a very critical role in orchestrating and perpetuating inflammation in many diseases including obesity, cancer and cachexia. The specific cytokine and chemokine modulators are now used in the treatment of obesity, cancer and cachexia. These cytokine modulators include Etanercept, Infliximab, Pentoxifylline and Thalidomide.These cytokine modulators are known to increase the patients body weight and muscle mass (42).

Anabolic agents: Anabolic modulators include various chemicals, very importantly steroids. These steroids mimics human androgens. They also known more properly asanabolic-androgenic steroids (AAS). They include natural androgens (testosterone)and synthetic androgens (17á-alkylated androgens, 1-methyl androgens, and nandrolone and its derivatives). These synthetic anabolic agents are structurally related and have similar effects ofnatural testosterone. Other non-steroidal anabolic agents include peptide hormone Insulin. Insulin also increases muscle mass and body weight (43).

Combination treatments : Here combination of various agents was used to treat cancer cachexia, so that overall quality and lifestylewill be modified for betterment of these patients. Example thalidomide + cinobufagin, Megestrol Acetate + Thalidomide etc (44).

Physical exercise : Physical exercise improves overall health of normal people as well as cancer associated cachexia patients. Therefore, it is very important to include physical exercise along with dietary and nutritional interventions approaches in the treatment option, increased physical activity and exercise with lifestyle modification has been proposed as another crucial component of the multidimensional approach to treat cancer cachexiapatients (45,46).

Physical exercises have lot of beneficial effects and it plays very important role in converting white adipose tissue (WAT) to brown adipose tissue (BAT) and they improve the overall health by suppressing adipose tissue inflammation and other associated factors in cachexia patients (47). Exercise also decreases overall adipose tissue mass but aids in sustaining and building new skeletal muscle mass by increasing adiponectin and other adipokines (48). Exercise also prevents damage of cells from oxidative stress (49). Therefore, cancer cachexia patients also get these benefits from regular exercise. Physical activity is also very effective in improving cachexia and cancer survivors overall health.

Conclusion

Cachexia is a very serious health condition which disturbs these patients in many ways including physically, emotionally and mentally and it is also a severe, life threatening and limiting

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complication (50). Passive loss of stored fat and muscle mass due to rapid growth of tumor and its metastasis is a major culprit (51). Along with this the secondary effect of these tumor burden include loss of appetite, nausea, vomiting may also play a big role in this complication (52). Therefore, this disease is a complex multifactorial disorder with numerous drivers involved in the development and progression. The complex associationof obesity, cancer and cachexia and related events are illustrated in the Fig. 1. These metabolic events includeunrestrained lipolysis, increased energy expenditure due to WAT to beige and BAT expansion and activation (48,49). It is very clear that all these molecular events are caused by rapid division of tumor cells and demonic growth of these tumor cells.Tumor cells eternal desire for energy and nutrition and their requirement of large quantity of biomolecules makes cancer cachectic patients to lose adipose tissue and muscle mass (53). To better understand the cross talk between obesity, cancer and cachexia there is an urgent need of more and more advanced research with the identification of unknown factors which plays major role in these diseases (54). Therefore, new studies will in turn aidin development of therapeutic interventions and to improve the quality of life from this deadly health condition. Overall, the hard-gained knowledge of pathophysiology of obesity and cancer associated cachexiamay provide novel therapeutic targets and optionsto ameliorate from obesity, cancer and cachexia and their metabolic complications.

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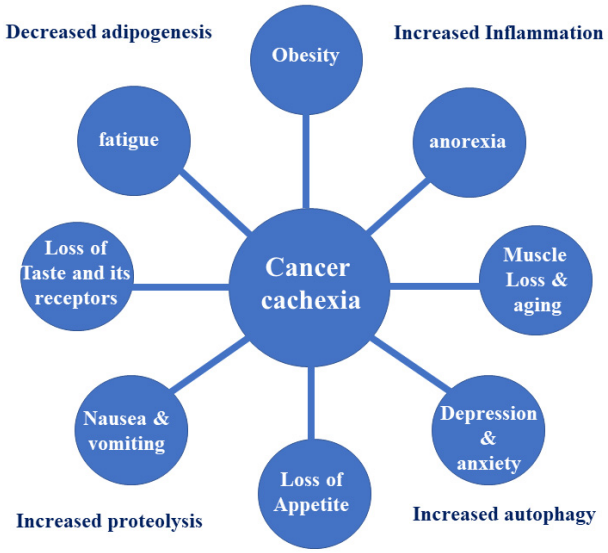

Figure 1: The complex symptoms and mechanisms of cancer cachexia

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