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Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2004). Lehninger Principles of Biochemistry, (4th edition), W.H. Freeman & Co., New York, USA, pp. 73-111.

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Synthesis of Methyl Esters by Transesterification Catalyzed by Cutinase from *Pseudomonas cepacia* NRRL B 2320 and Kinetic analysis

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Abstract

The synthesis of methyl esters by transesterification of tributyrin, triolein and soyabean oil in organic solvent was demonstrated using cutinase from *Pseudomonas cepacia* NRRL B-2320. The optimal temperature range for the enzyme catalyzed synthesis was found to be from 35°C to 40°C. The maximum conversion (65%) during synthesis of ester was obtained with 2.5 mg/ml of enzyme. The optimal methanol to oil ratio was found to be 1.5:1 with all three substrates. The kinetic analysis for the synthesis of methyl esters from tributyrin/triolein/ soyabean oil have shown that the Ping-Pong bi bi model with alcohol inhibition was found to be the most suitable for the prediction of the reaction kinetics for all the cases.

Keywords: Biodiesel, Cutinase, Ping-Pong bi bi model, *Pseudomonas cepacia,* Transesterification.

Introduction

Fatty acid esters of alcohols are of increasing economic interest in many industries involving a wide range of applications, *viz*., biocarburants, bio-surfactants, biolubricants, solvents, hydraulic and drilling fluids, dispersing agents, cosmetics (1), and biodiesel (2, 3). The scarcity in supply of oil and the growing requirement of fuels that have a lesser environmental effect brought into the attention towards the alternative energy sources, mostly liquid fuels for transportation. Recently, biodiesel is drawing the attention of researchers as an important alternative fuel in relation to the traditional fossil-based energy resources due to the increase in petroleum price, consumption of resources and to its environmental advantages (4-7). Biodiesel is thought to be a potential substitute for oil- derived fuels and its production has increased rapidly in the last few years owing to its compatibility with the existing engines. Biodiesel can also be operated in compression-ignition engines as petroleum diesel. Biodiesel is used either blended with petroleum diesel in ratios of 2%, 5%, or 20% or as pure biodiesel (8). Biodiesel fuels can be used in regular diesel vehicles without making any changes to the engines (9). The advantages of biodiesel over diesel fuel are that, it is oxygenated and act as better lubricant than diesel fuel. Thus, it increases the life of engines and combusted more completely. Furthermore, biodiesel offers lower emissions of greenhouse gases when compared to those given off by petroleum diesel (10). The amount of particulate matter produced by biodiesel is also considerably less. Biodiesel is mainly composed of fatty acid alkyl esters. Biodiesel is commonly produced by transesterification of oils with short-chain alcohols. It involves the transformation of triglycerides into fatty acid alkyl ester, in the presence of an alcohol, such as methanol or ethanol, with the help of catalyst, such as an alkali or acid (11). Currently, it is produced by many industries through a homogeneous alkaline

catalytic method. This method however, employed the use of strong bases as catalysts, thus raising some additional environmental issues (3). In comparison to the direct synthesis of esters from oil and alcohols, enzymatic means has been suggested to be a good alternative; due to the enzymatic catalysis provides an energy-saving procedure with high selectivity. Enzymatic catalysis endorses an alternative route that will allow the production of biodiesel using mild reaction conditions, which is also beneficial in regard to the environmental issues. Moreover, separation and purification of biodiesel fuel produced by enzymatic transesterification is easier than produced by chemical method (12). In the overall enzymatic reaction between one molecule of triglyceride (TG) and three molecules of an alcohol (Al), a molecule of glycerol and three molecules of fatty acid alkyl esters (AE) are produced, which incorporate biodiesel. Several reports are available on the biodiesel production using lipase (3, 6, 10, 13-15). Cutinase also have been proved to be a very promising catalyst (4, 16). Cutinase is a hydrolytic enzyme with lypolytic activity and was observed to efficiently catalyze the triglyceride hydrolysis and transesterification reaction in homogeneous as well as reverse micellar medium. Recently, few studies on production of methyl/ethyl ester of oil mixtures have also been carried out using cutinase (4, 16).

In this investigation, we have studied the *P. cepacia* cutinase catalyzed synthesis of methyl esters of tributyrin, triolein and soyabean oil in isooctane as a solvent under various conditions. Here, we have also studied the kinetics for the synthesis of methyl esters of these oils in isooctane. This is first report on transeseterification soyabean oil and tributyrin catalyzed by *P. cepacia* cutinase.

Materials and Methods

Methanol, triolein, tributyrin, methyl oleate, methyl palmitate and methyl linoleate, lipase (*Pseudomonas sp*) were purchased from sigma. Isooctane (Merck) was dried over 4 Å molecular sieve. Tris (hydroxymethyl) amino methane (Tris) was also purchased from Sigma. The cutinase enzyme used for reaction was isolated from *Pseudomonas cepacia* NRRL B 2320 (also known as *Burkholderia cepacia*). The complete method of production and purification of cutinase from *P. cepacia* NRRL B 2320 has been described previously (17, 18).

Enzymatic synthesis of methyl esters: In the standard protocol, ester synthesis was carried out in 10 ml screw-capped test tubes as bioreactors. Unless otherwise specified 0.1mM of enzyme in lyophilized powder form (calculated on the basis of molecular mass of enzyme as obtained from SDS PAGE was 26.5kDa (18) was added to the vial containing triglycerides/oil and methanol in 2.5 ml of isooctane. The tubes were kept in a horizontal shaker at 37°C. Initial velocities were determined in each case by taking samples of the medium and analyzed using GC chromatography.

GC analysis of esters : Synthesis of fatty acid ester was analyzed by gas chromatograph (Varian-450) (17). The samples were withdrawn at regular time intervals (100 µl), diluted to 1 ml with isooctane, filtered through 2 micron filter and 1 µl of the reaction mixture was injected into CPSIL 8CB column and compounds were detected by Flame Ionization Detector (FID). The injector and detector temperature were set at 250°C. The temperature programmed for different esters are given below,

Methyl butyrate: 150°C for (0.5min) -15°C/min-250°C (10min); tR 1.69min
Methyl palmitate: 150°C for (0.5min) -30°C/min-250°C (10min); tR 3.14 min
Methyl oleate: 150°C for (0.5min) -30°C/min-250°C (10min); tR 4.88 min
Methyl linoleate: 150°C for (0.5min) -30°C/min-250°C (10min); tR 2.67 min

***Kinetic study of transesterification catalyzed by cutinase*:** Originally Michaelis–Menten equation was derived for kinetics of single substrate reaction. However, a reaction involving two substrates may also thought to obey the

Michaelis–Menten kinetics, if the reaction rate depends on the concentration of both the substrates, so that if one substrate concentration varied while other maintaining constant, the reaction behaves like a single substrate reaction obeying Michaelis–Menten kinetics. The experimental plan for kinetic study of transesterification of tributyrin, triolein and soyabean oil catalyzed by cutinase is given in the Table 1. Previously, lipase catalyzed transerification reactions have been described by Ping-Pong bi bi kinetic model equation 1 (19).

$$v = \frac{V_{\max}}{1 + \frac{K_{mA}}{[A]} + \frac{K_{mB}}{[B]}}$$

Where [A] and [B] are the initial concentration of triglyceride and methanol respectively, where *v* is the initial reaction, V_{max} is the maximum reaction rate, K_{mA} and K_{mB} are the Ping-Pong constants for the triglyceride (*A*) and the methanol (*B*), respectively.

The competitive inhibition by alcohol (1) or triglyceride or both alcohol and triglyceride leads to the use of following modified Michaelis–Menten equations (17).

$$v = \frac{V_{\max}}{1 + \frac{K_{mA}}{[A]}\left(1 + \frac{[B]}{K_{IB}}\right) + \frac{K_{mB}}{[B]}} \quad (2)$$

6 Here, K_{IB} is the inhibition constant for alcohol

$$v = \frac{V_{\max}}{1 + \frac{K_{mB}}{[B]}\left(1 + \frac{[A]}{K_{IA}}\right) + \frac{K_{mA}}{[A]}} \quad (3)$$

7 Here, K_{IA} is the inhibition constant for triglyceride,

$$v = \frac{V_{\max}}{1 + \frac{K_{mA}}{[A]}\left(1 + \frac{[B]}{K_{IB}}\right) + \frac{K_{mB}}{[B]}\left(1 + \frac{[A]}{K_{IA}}\right)} \quad (4)$$

Results and Discussion

***Effect of enzyme concentration*:** Fig. 1 shows the effect of cutinase concentration on the conversion (%) of methyl ester of tributyrin and triolein. The conversion (%) increased with the increasing concentration of cutinase from 0.5 mg/ml to 2.5 mg/ml in the reaction mixture. Further increase of cutinase concentration did not show any significant increase in methyl ester conversion for both tributyrin and triolein. The maximum conversion achieved for methyl butyrate and methyl oleate was found to be 65%, and 30%, respectively. Cutinase from *P. cepacia* showed higher affinity towards shorter chain length substrate, so the conversion % has decreased with increase in fatty acid chain length. In this study, reactions were carried out with 1-3% cutinase (w/w of oil). Xie and Ma, 2010 (3) reported 45% conversion for transesterification of oil with methanol using 40% lipase (w/w). Tamalampudi et al., 2008 (20) and Martin and Otero, 2008 (21) achieved 75% conversion for methanolysis of oil with 10% (w/w of oil) and 50% (w/w of oil) lipase, respectively. Badenes et al., 2010 (4) observed 70% of conversion during the transesterification of triolein with 1-2 % cutinase (w/w of oil).

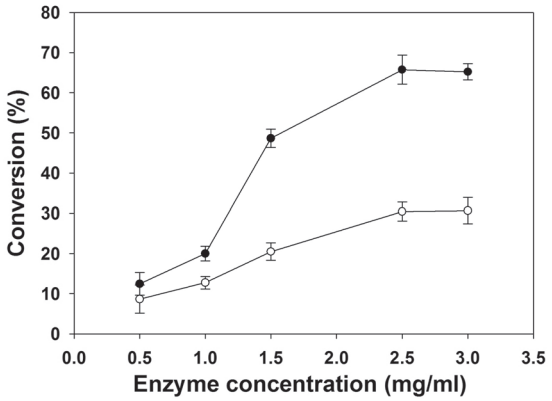

Fig. 1. Effect of cutinase concentration on conversion of methyl butyrate (•) and methyl oleate (o) during cutinase catalysed transesterification of tributyrin and triolein. (The values are average of triplicates and standard errors bars shown in figure are in 95% confidence level)

***Effect of temperature on methyl ester synthesis*:** The effect of temperature on cutinase produced in this study and commercial lipase (sigma) activity was evaluated in the range of 25–50°C for transesterification reaction of

tributyrin and triolein with methanol (Fig. 2). It was observed that the stability of cutinase was better than that of the lipase. The maximum conversion was obtained at 37°C for cutinase, but it did not decrease much up to 45°C. Where as in case of lipase, the maximum conversion obtained at 35°C, but it decreased when temperature increased above 40°C. The methyl ester conversion was higher for transesterfication of tributyrin than triolein with cutinase, whereas reverse was true for the lipase. This may be because cutinase have higher affinity towards short-chain length fatty acids (17), while lipase was active on longer chain length fatty acids. The conversion during the transesterification of triolein by cutinase was almost comparable to the commercial lipase. The cutinase from *Fusarium solani* showed optimum transesterification activity at 30°C (4).

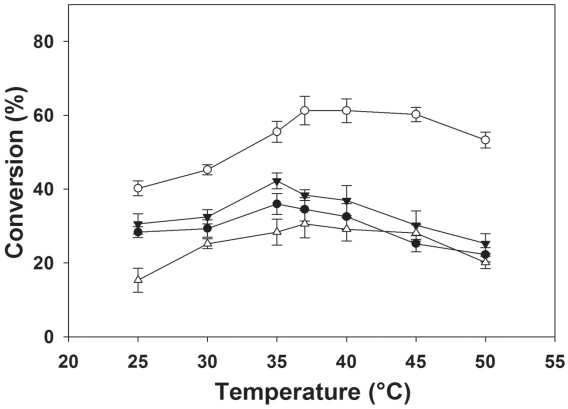

Fig. 2. Effect of temperature on the conversion of methyl butyrate by lipase (•) and cutinase (o), and methyl oleate by lipase (▼) and cutinase (Δ). (The values are average of triplicates and standard errors bars shown in figure are in 95% confidence level)

***Effect of substrate molar ratio*:** Molar ratio of methanol to oil is one of the important parameters affecting the enzymatic transesterification reaction. Fig. 3 and 4 shows the change of methyl butyrate/oleate conversion with time as a function of molar ratio of methanol/oil. The enzymatic transesterification reaction was carried out at 37°C for 30 h, with methanol/oil molar ratio of 0.5:1, 1:1, 1.5:1, 2:1 and 3:1. As indicated in Fig. 3, the conversion to methyl butyrate increased with increase in molar ratio of methanol/tributyrin from 0.5:1 and reached the maximum value (66.52%) at molar ratio of 1.5:1. Further increase in molar ratio from 1.5:1 to 3:1 resulted decrease in conversion (35.45%). A similar trend (Fig. 4) was observed during the transesterification reaction of triolein and methanol. A maximum of 36% conversion of methyl oleate could be achieved after 24 h with methanol/triolein ratio of 1.5:1. Salis et al., 2005 (10) observed about 40% conversion of methyl ester during transesterification of triolien catalysed by *P. cepacia* lipase. Experiments were also carried out with commercial lipase. It was also observed from the Fig 5 and 6 that transesterification activity of lipase was also decreased when methanol/oil ratio increases beyond 1.5:1. In this

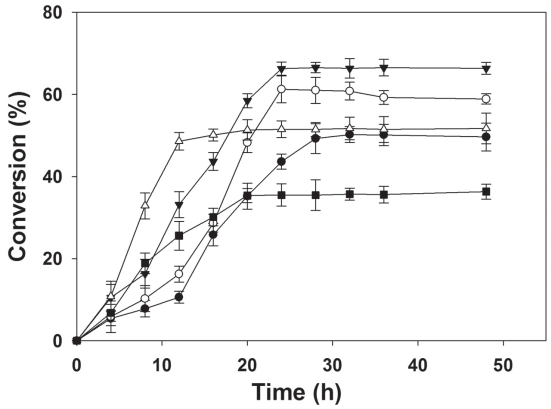

Fig. 3. Effect of molar ratio (methanol/tributyrin (•) 0.5:1, (o) 1:1, (▼) 1.5:1, (Δ) 2:1, (■) 3:1) on the conversion of methyl butyrate during transesterification of tributyrin with methanol catalyzed by cutinase. (The values are average of triplicates and standard errors bars shown in figure are in 95% confidence level)

experiment, the efficiency of lipase was less than cutinase for the transesterification of tributyrin. Increase in molar ratio of methanol/oil beyond 1.5:1 decreased the conversion to methyl esters catalyzed by lipases/cutinases was also reported (4, 22). It is clear that any molar ratio of methanol

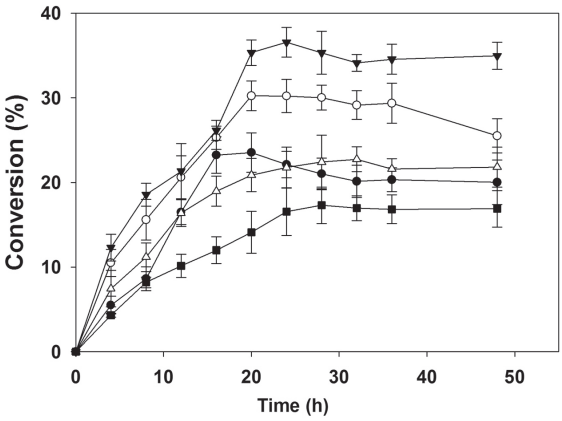

Fig. 4. Effect of molar ratio (methanol/triolein (•) 0.5:1, (o) 1:1, (▼)1.5:1, (Δ) 2:1, (■) 3:1) on the conversion of methyl oleate during transesterification of triolein with methanol catalyzed by cutinase. (The values are average of triplicates and standard errors bars shown in figure are in 95% confidence level)

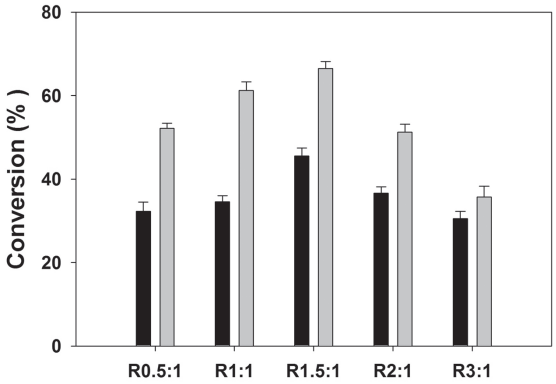

Fig. 5. Effect of molar ratio (methanol/tributyrin) on conversion of methyl butyrate during transesterification of tributyrin with methanol catalyzed by lipase (■) and cutinase (▣). (The values are average of triplicates and standard errors bars shown in figure are in 95% confidence level)

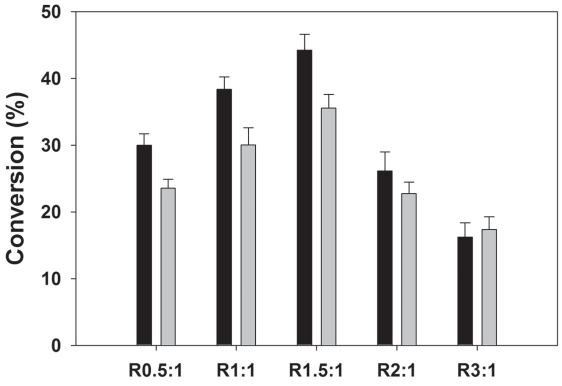

Fig. 6. Effect of molar ratio (methanol/triolein) on conversion of methyl oleate during transesterification of triolein with methanol catalysed by lipase (■) and cutinase (▣). (The values are average of triplicates and standard errors bars shown in figure are in 95% confidence level)

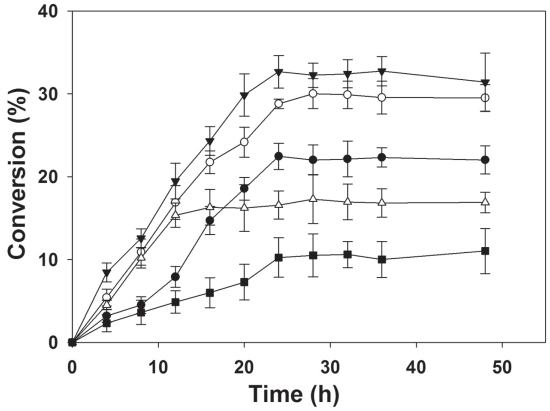

Fig. 7. Effect of molar ratio (methanol/Soyabean oil (•) 0.5:1, (o) 1:1, (▼)1.5:1, (Δ) 2:1, (■) 3:1) on the conversion of methyl ester of oil during transesterification of Soyabean oil with methanol catalyzed by cutinase. (The values are average of triplicates and standard errors bars shown in figure are in 95% confidence level).

to oil above 1.5:1 could lead to the deactivation of the enzyme. Owing to the low solubility of methanol in the oil; excessive methanol might decrease the enzyme activity by the contact with insoluble methanol, which exists as drops in the oil (23). This inactivation of enzyme at high methanol to oil molar ratio may be rectified by immobilizing the enzyme (3, 24) or step wise addition of methanol to the reaction media (3, 25). Experiments were also carried out for transesterification of soybean oil catalysed by cutinase from *P. cepacia*. The soyabean oil used

in this study has the following composition as given by the manufacturer; *viz*., palmitic acid 11%, stearic acid 4%, oleic acid 24%, linoleic acid 54% and linolenin acid 7%. The average molecular weight calculated as 879.87g mol^{-1}. The enzymatic transesterification reaction was carried out at 37°C, with methanol/oil molar ratio of 0.5:1, 1:1, 1.5:1, 2:1 and 3:1 (Fig. 7). It was observed that with increase in molar ratio from 0.5:1 to 1.5:1, the maximum conversion at 24 h increased from 22.45% to 32.66%, but further increase in the molar ratio resulted in reduction of conversion to 10% due to inhibition of the enzyme by methanol. The reaction efficiency was also compared with commercial lipase (Fig 8). The conversion (%) of methyl esters of soyabean oil by cutinase was comparable with the commercially available lipase and also other reported lipases. Xie and Ma, 2010 (3) reported 45% conversion of methyl esters during transesterification of soyabean oil catalysed by lipase with 1.5:1 methanol/ soyabean oil ratio. The increase in methanol/ soyabean oil ratio to 3:1 resulted in the decrease in conversion to 5%. Yang et al., 2009 (26) observed that the conversion of 40% and 20% methyl ester after 24 h incubation during transesterification catalyzed by recombinant *P. fluorescence* lipase 26-2 and lipase AK. Similar type of alcohol inhibition was also observed by Du et al., 2004 (25) for transeseterification of soyabean oil.

Kinetic study of synthesis of methyl ester of Tributyrin and Triolein *:* The effect of different concentrations of tributyrin (Fig. 9a) or methanol (Fig. 9b) on the initial rates of methyl butyrate synthesis was studied in the range of 0 - 0.5 M for both the substrates. The effect of increasing tributyrin concentration on initial reaction rate at three different concentrations of methanol (0.05 M, 0.1 M, 0.2 M) have shown in Fig. 9(a) and Fig. 9(b) showed the effect of increasing methanol concentration on initial reaction at three

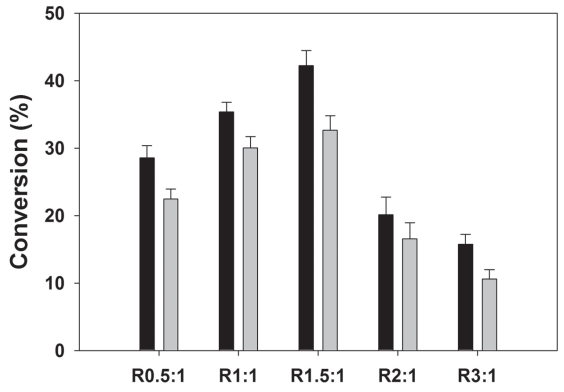

Fig. 8. Effect of molar ratio (methanol/soyabean oil) on conversion of methyl ester during transesterification of soyabean oil with methanol catalyzed by lipase (■) and cutinase (■). (The values are average of triplicates and standard errors bars shown in figure are in 95% confidence level)

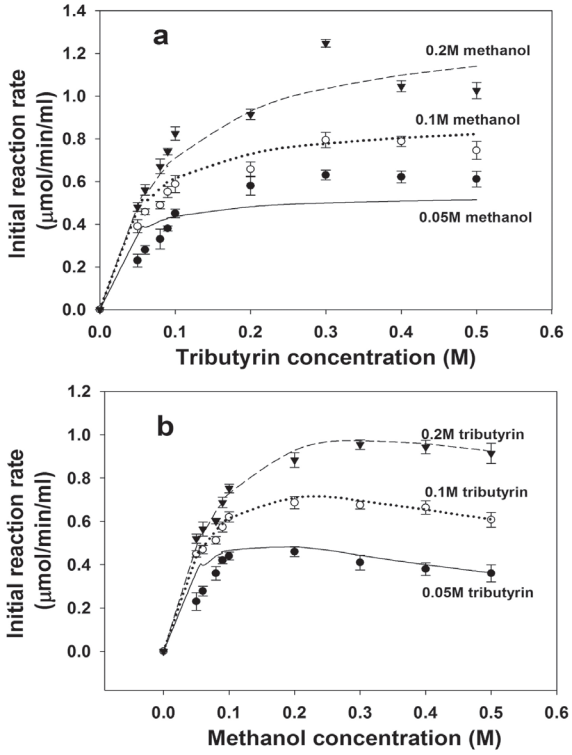

Fig. 9. Initial reaction rate as a function of (a) tributyrin or (b) methanol concentrations. The experimental data are fitted to a Ping-Pong kinetic model with alcohol inhibition Eq. (2). The parameter values in Table 1 are used to calculate the lines shown. (The values are average of triplicates and standard errors bars shown in figure are in 95% confidence level).

different concentration of tributyrin (0.05 M, 0.1 M, and 0.2 M). Different kinetics models given by the eq. 1, 2, 3 and 4 were used to fit the experimental data for both the cases. The kinetic parameters estimated using these equations were given in Table 2. Eq. 1 is expressed based on Michaelis-Menten equation for two substrates Ping–Pong bi bi kinetic model. But as seen in Fig. 9, this model may not predict the kinetics of the reaction appropriately after certain concentration of substrates, where increasing concentration of one or both substrates has inhibitory effect on reaction rate. So, the introduction of one or more extra terms may be useful for proper prediction of the reaction kinetics with inhibitory effect of one or more substrates. K_{IB} and K_{IA} are inhibition constant for methanol and tributyrin/triolein, respectively. In eq. 2 and eq. 3 are obtained when inhibition due to methanol or tributyrin/triolein are considered, where as eq. 4 has both K_{IA} and K_{IB} considering the inhibition by both the substrates. The results obtained by fitting the experimental data to the above mentioned four equations, it was observed that kinetic parameters did not vary much for the other three equations than the classical Michaelis-Menten equation. From the all four models tested, the best fit was obtained with the model eq. 2. The lines shown in the Fig. 9(a) and 9b were obtained using the values given by eq. 2 in Table 2. It was observed that there was strong alcohol inhibition during transesterification reaction of tributyrin within the experimental ranges of methanol and tributyrin (Fig. 9). From the R^2 value of the different models it could also be observed that the eq. 2 has highest R^2 (0.918) among the all tested models. Similar experiments were conducted for synthesis of methyl oleate by transesterification of triolein with methanol (shown in Fig. 10(a) and 10(b). The experimental data were fitted to the eq. 1, 2, 3 and 4 and the estimated kinetic parameters were presented in Table 3. The lines shown in the Fig. 10(a) and 10(b) were calculated using the parameters obtained from eq. 2, in Table 3. From the decreasing profile of initial reaction rate with increasing methanol concentration during the transestrification of triolein catalysed by *P. cepacia*, the strong alcohol inhibition was observed, and eq. 2 was found to be the best fitted model in this case as well. Previously, Badenes et al., 2010 (4) also reported that the similar kind of alcohol inhibition during the transesterification reaction of triolein with methanol catalysed by *Fusarium solani* cutinase. They observed that decrease in methyl ester conversion from 77% to 20% at 24 h, when methanol concentration increased from 390 mM to 590 mM. Several reports are also available on the inhibition effect of alcohol on transesteriffication reaction catalysed by lipase (4).

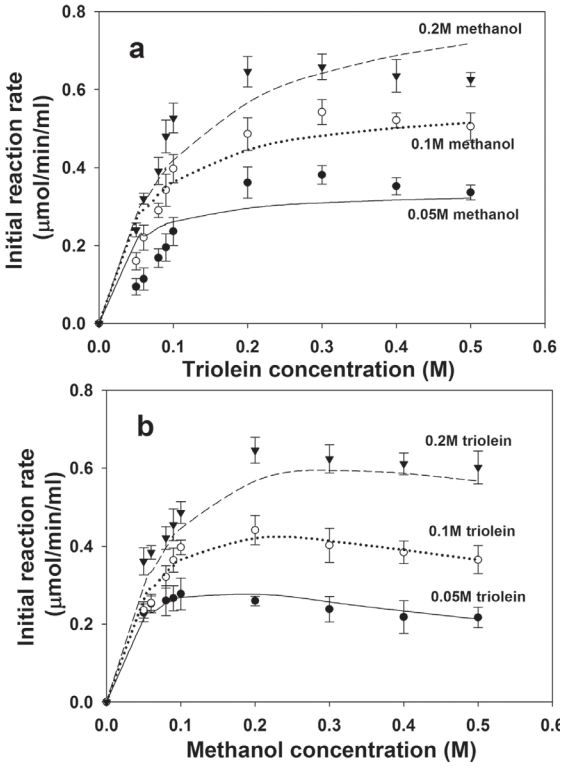

Fig. 10. Initial reaction rate as a function of (a) triolein or (b) methanol concentrations. The experimental data are fitted to a Ping-Pong kinetic model with alcohol inhibition Eq. (2). The parameter values in Table 2 are used to calculate the lines shown. (The values are average of triplicates and standard errors bars shown in figure are in 95% confidence level).

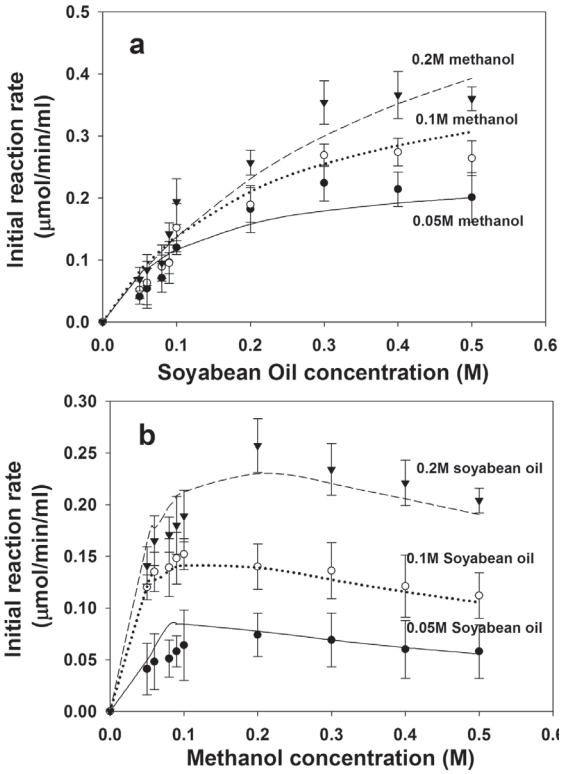

Fig. 11. Initial reaction rate as a function of (a) Soyabean oil or (b) methanol concentrations. The experimental data are fitted to a Ping-Pong kinetic model with alcohol inhibition Eq. (2). The parameter values in Table 3 are used to calculate the lines shown. (The values are average of triplicates and standard errors bars shown in figure are in 95% confidence level).

The kinetics of transesterification reaction catalyzed by *P.cepacia* cutinase was studied with the different concentrations of soyabean oil and methanol. The kinetic parameters calculated using the eq. 1, 2, 3 and 4 is shown in Table 4. Fig 11 showed that the variation in initial reaction rate as the function of substrate (soyabean oil or methanol) concentration. The strong methanol inhibition could be observed in this case also. The best result (on the basis of R^2 0.931) was obtained when experimental data were fitted to eq. 2. The lines shown in Fig 11a and 11b were obtained by using the parameters given in Table 4 with eq 2.

Conclusion

Cutinase from *P. cepacia* NRRL B 2320 was found to be one of the potential industrial enzymes to perform transesterification of tributyrin, triolein and soyabean oil with methanol. The optimum conversion have achieved with 2.5mg/ml of protein. The maximum conversion of methyl butyrate and oleate was achieved at 37°C, after 24 h of transesterification. The optimum molar ratio for methanol to tributyrin/ triolein was found to be 1.5:1. The higher concentration of methanol causes the decrease in conversion of methyl butyrate/oleate. The kinetic analysis of the transesterification reaction catalysed by *P. cepacia* cutinase showed the strong alcohol inhibition for both tributyrin and triolein. The V_{max} for tributyrin, triolein and soyabean oil by transesterification was found to be 2.678, 1.838, 1.5 µmol min^{-1} ml^{-1}, respectively with the alcohol inhibition model.

Table 1. Experimental plan for kinetic study of transesterification of tributyrin, triolein and soyabean oil catalyzed by cutinase

Concentration of tributyrin/ triolein/soyabean oil (A) (M)	Concentration of methanol (B) (M)
0.05-0.5	0.05
0.05-0.5	0.1
0.05-0.5	0.2
0.05-0.5	0.5
0.1	0.05-0.5
0.2	0.05-0.5

Acknowledgement

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References

1. Dossat, V., Combes, D. and Marty, A. (2002). Lipase-catalysed transesterification of high oleic sunflower oil. Enzyme Microb Technol, 30:90-94.

Table 2 : Estimated kinetic constants for the *P. cepacia* cutinase-catalyzed transesterification of tributyrin (A) with methanol (B) using different kinetic models

	V_{max} (µmol/min/ml)	K_{mA}(M)	K_{mB} (M)	K_{IA}(M)	K_{IB}(M)	R^2
Eq. 1	2.237	0.141	0.138	—-	—-	0.904
Eq. 2	2.678	0.097	0.198	—-	0.24	0.918
Eq. 3	2.655	0.206	0.118	0.80	——	0.896
Eq. 4	3.729	0.216	0.176	0.55	0.35	0.838

Table 3 : Estimated kinetic constants for the *P. cepacia* cutinase-catalyzed transesterification of triolein (A) with methanol (B) using different kinetic models

	V_{max} (µmol/min/ml)	K_{mA}(M)	K_{mB} (M)	K_{IA}(M)	K_{IB}(M)	R^2
Eq. 1	1.528	0.179	0.156	——-	——	0.881
Eq. 2	1.838	0.137	0.222	——-	0.31	0.897
Eq. 3	1.841	0.28	0.099	0.414	——	0.852
Eq. 4	2.464	0.235	0.204	0.81	0.35	0.83

Table 4 : Estimated kinetic constants for the *P. cepacia* cutinase-catalyzed transesterification of soyabean oil (A) with methanol (B) using different kinetic models

	V_{max} (µmol/min/ml)	K_{mA}(M)	K_{mB} (M)	K_{IA}(M)	K_{IB}(M)	R^2
Eq. 1	1.331	0.723	0.191	——	——	0.902
Eq. 2	1.501	0.546	0.253	——	0.351	0.931
Eq. 3	1.60	0.928	0.185	1.4	——	0.911
Eq. 4	1.5	0.632	0.133	0.55	0.41	0.907

2. Aulakh, S.S., Chhibber, M., Mantri, R. and Prakash, R. (2011). Whole cell catalyzed esterification of fatty acids to biodiesel using Aspergillus sp. Biocatal Biotransform, 29: 354-358
3. Xie, W. and Ma, N. (2010). Enzymatic transesterification of soybean oil by using immobilized lipase on magnetic nano-particles. Biomass Bioenergy, 34:890-896.
4. Badenes, S.M., Lemos, F. and Cabral, J.M.S. (2010). Transesterification of oil mixtures catalyzed by microencapsulated cutinase in reversed micelles. Biotechnol Lett, 32:399-403.
5. Korbitz, W. (1999). Biodiesel production in Europe and North America, an encouraging prospect. Renew Energ, 16:1078-1083.
6. Ma, F.R. and Hanna, M.A. (1999). Biodiesel production: a review. Bioresour Technol, 70:1-15.
7. Manzanera, M., Molina-Munoz, M.L. and Gonzalez-Lopez, J. (2008). Biodiesel: an alternative fuel. Recent Pat Biotechnol, 2:25-34.
8. Shen, X. and Vasudevan, P.T. (2008). Transesterification of Waste Olive Oil by *Candida* Lipase. Bull Sci Technol Soc, 28:521-528.
9. Anastopoulos, G., Lois, E., Serdari, A., Zanikos, F., Stournas, S. and Kalligeros, S. (2001). Lubrication properties of low-sulfur diesel fuels in the presence of specific types

of fatty acid derivatives. Energy Fuels, 15:106-112.

10. Salis, A., Pinna, M., Monduzzi, M. and Solinas, V. (2005). Biodiesel production from triolein and short chain alcohols through biocatalysis. J Biotechnol, 119:291-299.

11. Hoydoncx, H.E., De Vos, D.E., Chavan, S.A. and Jacobs, P.A. (2004). Esterification and transesterification of renewable chemicals. Top Catal, 27:83-96.

12. Ranganathan, S.V., Narasimhan, S.L. and Muthukumar, K. (2008). An overview of enzymatic production of biodiesel. Bioresour Technol, 99:3975-3981.

13. Xie, W. and Ma, N. (2009). Immobilized lipase on Fe3O4 nanoparticles as biocatalyst for biodiesel production. Energy Fuels, 23:1347-1353.

14. Xie, W. and Wang, J. (2011). Immobilized lipase on magnetic chitosan microspheres for transesterification of soybean oil. Biomass Bioenerg, 36:373-380.

15. Coggon, R., Vasudevan, T.P. and Sanchez, F. (2007). Enzymatic transesterification of olive oil and its precursors. Biocatal Biotransform, 25:135-143

16. Badenes, S.M., Lemos. F. and Cabral, J.M.S. (2011). Stability of cutinase, wild type andmutants, in AOT reversed micellar system – effect of mixture components of alkyl esters production. J Chem Technol Biotechnol, 86:34-41.

17. Dutta, K and Dasu, V.V. (2011). Synthesis of short chain alkyl esters using cutinase from *Burkholderia cepacia* NRRL B2320. J Mol Catal B Enzym, 72:150-156.

18. Dutta, K., Hegde, K. and Dasu V.V. (2013). Novel cutinase from *Pseudomonas cepacia* NRRL B-2320: Purification, characterization and identification of cutinase encoding genes. J Gen Appl Microbiol, (In press).

19. Paiva, A.L., Balcao, V.M. and Malcata, F.X. (2000). Kinetics and mechanisms of reactions catalyzed by immobilized lipases. Enzyme Microb Technol, 27:187-204.

20. Tamalampudi, S., Talukder, M.R., Hama, S., Numata, T., Kondo, A. and Fukuda, H. (2008). Enzymatic production of biodiesel from Jatropha oil: a comparative study of immobilized-whole cell and commercial lipases as a biocatalyst. Biochem Eng J, 39:185-189.

21. Martin, H.E. and Otero, C. (2008). Different enzyme requirements for the synthesis of biodiesel: Novozym (R) 435 and Lipozyme (R) TL IM. Bioresour Technol, 99:277-286.

22. Shimada, Y., Watanabe, Y., Sugihara, A. and Tominaga, Y. (2002). Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. J Mol Catal B Enzym, 17:133-142.

23. Watanabe, Y., Shimada, Y., Sugihara, A. and Tominaga, Y. (2001). Enzymatic conversion of waste edible oil to biodiesel fuel in a fixed-bed bioreactor. J Am Oil Chem Soc, 78:703-707.

24. Li, S.F., Fan, Y.H., Hu, R.F. and Wu, W.T. (2011). Pseudomonas cepacia lipase immobilized onto the electrospun PAN nanofibrous membranes for biodiesel production from soybean oil. J Mol Catal B Enzym, 72:40- 45.

25. Du, W., Xu, Y., Liu, D. and Zeng, J. (2004). Comparative study on lipase-catalyzed transformation of soybean oil for biodiesel production with different acyl acceptors. J Mol Catal B Enzym, 30:125-129.

26. Yang, J., Zhang, B. and Yan,Y. (2009). Cloning and expression of *Pseudomonas fluorescens* 26-2 lipase gene in *Pichia pastoris* and characterizing for transeseterification. Appl Biochem Biotechnol, 159:355-365.

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DNA Sequencing Analysis Software for Sanger Data-Sets: Comparisons of Basic features Useful for Mutational Studies

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Abstract

Mutation analysis is based on the alignment and comparison of the fluorescent traces produced by Sanger DNA sequencing. Various secondary analysis sequencing software tools are available for scientific research, but as the commercial applications of genetic analysis began to grow, sequencing analysis software tools have expanded their features and uses. This paper explores software programs such as CodonCode Aligner, Geneious and Sequencher which includes sequence assembly, contig editing, and mutation detection and labeling. In an attempt to assess their features for the benefit of users we have evaluated the features based on some specific criteria. The features compared include chromatogram editing, database creation and management, quality assessment, SNP statistics, and other features useful for simple sequence analysis. These features are discussed with respect to the three platforms to gain insight into the specific areas for which they are most useful. Our comparative analysis of three software programs revealed that all the basic features required for muatiaonal analysis are similar to a few unique features in each programme. Hence end user needs to select the appropriate program.

Key Words: Mutational analysis, Sequencing software and Sanger sequencing.

Introduction

Sanger sequencing still remains as a corner stone to interrogate genes for mutations including substitutions, indels (deletion and insertion of the bases) in many laboratories despite rapid advances in sequencing technology (1). Sanger sequencing involves synthesis of DNA chains of varying lengths and these fragments are then separated by capillary tube electrophoresis, a method in which an electric field pulls molecules across a hair like capillary fiber. Further downstream analysis including both primary and secondary analysis is usually followed to conclude the analysis wherein primary analysis tools usually are included as default settings in the sequencing platforms. They translate the collected color-data images into the corresponding nucleotide bases and also perform basic functions such as basecalling, mobility shift correction and quality value. Secondary analysis tools further refine sequencing results with the possibility of mutation detection and genotyping, and production of graphical outputs. These secondary analysis tools are generally available in the from of third party softwares, *viz* (2-4).

It is a required task for experienced and inexperienced end users from biological labs to analyze sequences routinely and rapidly, hence the secondary analysis tools need to have a user-friendly interface. There are many such commercial software programs available, such as CodonCode Aligner, Geneious, Sequencher,

SeqMan Pro etc. with various features; however, there have been very few attempts to evaluate these programs for new user (5). In order to make features clearly available for a general user, this paper explores three analysis platforms in order to compare and evaluate the different specifications and features available for mutation analysis. It is also acknowledged that there are other more intricate ways of comparing different platforms, often involving complex algorithms. However, this study does not make use of such techniques because this study is meant to be a quick reference for the biologist who wishes to determine the bioinformatics software most suited for mutational analysis. The three platforms explored, are CodonCode Aligner (v4.1), Geneious (v6.1.7, created by Biomatters) and Sequencher (v5.2), which are all programs involving sequence assembly, contig editing, and mutation detection and labeling, available for both Windows and Mac OS X. In assessing the features provided by each software program, it is important not only to address tools needed in secondary analysis of sequences, but also to make sure that the programme is easy to use.

Materials and Methods

All the programs were installed on a computer running Windows 7. The three software programs are:

1. CodonCode Aligner, a program for sequence assembly, contig editing, and mutation detection, produced by CodonCode Corporation with the purpose of providing user-friendly, affordable software for DNA sequence assembly and alignment (2).
2. Geneious is a DNA, RNA and protein sequence alignment, assembly, automatic internal database creation and analysis software program (3).
3. Sequencher is a DNA sequencing program provided by Gene Codes Corporation (4).

***Criteria for selection of features of four software programs*:** The scope of the investigation is limited to basic features specific to simple gene mutation analysis. Any other features present, which are not explicitly related to gene analysis are not discusses. All three programs used for comparison were in demo mode. Hence, the features which are active in demo as well as paid versions of the programs were selected. Further, the features that are discussed in this paper have been chosen either as features necessary for DNA analysis, and/or as features that would be user-friendly for gene analysis. Thus, the three software programs have been evaluated and tested on the following features:

1. Chromatogram editing and assembly involve contig assembly, trim/clip ends, editing, and trace view features. This is one of the most basic and crucial features for a software program when analyzing genetic sequences.
2. Reference mapping/Alignment takes care of alignment of the various samples in a contig, and the ability to compare the samples in an alignment view.
3. Internal Database Creation and Management helps in automatic creation of personal projects. 4. Indication of quality of sample feature allows the user to assess the quality of a particular sample, usually indicated by different shades of highlighting of the bases. Sequence quality scoring has become an important issue due to the relatively low quality of raw data from most sequencing platforms.
5. Variant Detection is a manual feature in which nucleotide bases are analyzed against reference sequence and able to find out the variations.
6. SNP Finder and Analysis refers to a feature that when applied, automatically locates and analyzes all the SNPs in a given sample. It often shows statistics such as the percentage match between a sample and a reference, the location of all the SNPs, and, if applicable, the type.

7. Phylogenetics involves the reconstruction of evolutionary relationships and studying the patterns of relationships between organisms, based on DNA sequences. It usually includes a 'Build Tree' feature.
8. Protein Translation View translates the bases in a sequence to their corresponding amino acids.
9. Primer Design and Analysis allows design and editing of primers.
10. Bioinformatics Databases feature has access to large bioinformatics databases, either via connection to the internet, or within the program.
11. Application Programme Interface (API), a plug-in development kit, comments on the availability of further features and plug-ins for each software program.
12. Layout is evaluated based on the different viewing windows available, and whether the program's layout is easy to use and conducive to genetic analysis. Apart from these general features, overall ease of use is also assessed, followed by a general comment on the system compatibility and cost of software.

The above features are further discussed in relation to their uses and availability with reference to specific software programs in the Discussion section.

Selection of sequence reads for analysis: In order to evaluate these features, PCR products (amplicons of exons) of various genes were compared. These PCR products were from various patients who were referred for Diagnostic purposes with clinical suspicion of single gene disorders. The patient samples were collected with the prior consent of patients. The sequences of different qualities were taken in order to ensure that they are controlled when comparing features. The different qualities included: 1. Ten electropherograms of amplicon size ranging from 80 to 673, with relatively high interference (Fig. 1), 2. Ten electropherograms of amplicon lengths ranging from 82 to 199 with relatively less interference (Fig. 2), and 3. Ten electropherograms of amplicon lengths ranging from 64 to 242 with no interference (Fig. 3).

Results and Discussion

Comparison of the availability of features required for simple mutational analysis and their ease of use of all the programs are given in Table 1. In evaluating these features, a ✓ symbol indicates that a feature is present in the program, and a × symbol indicates that the feature is absent from the program. When applicable,

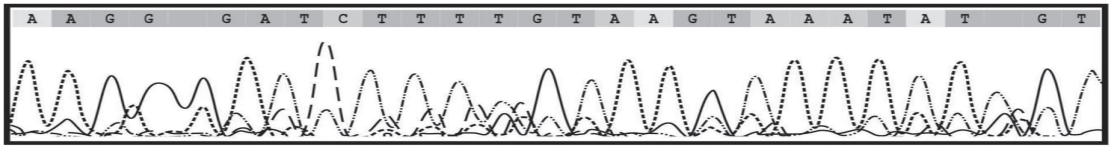

Fig. 1: Electropherogram with relatively high interference. All four nucleotide peaks are represented as follows: A (·····), G (—), C (– –) and T (– · · –).

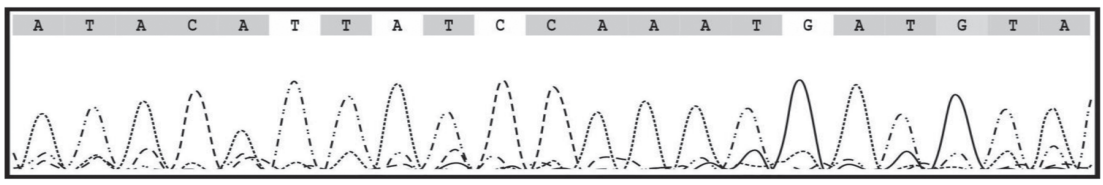

Fig. 2: Electropherogarm with relatively less interference. All four nucleotide peaks are represented as follows: A (·····), G (—), C (– –) and T (– · · –).

Table 1. Comparison grid of three different sequencing analysis softwares.

Features	Codon Code	Aligner Geneious	Sequencher
Chromatogram editing and assembly	✓ +++	✓ +++	✓ +++
Reference Mapping/AlignmentInternal	✓ +++	✓ +	✓ +
Database Creation and Management	×	✓	✓
Indication of Quality of Samples	✓	✓	×
Variant Detection	✓ +++	✓ ++	✓ +
SNP Finder and Analysis	✓ +	✓ ++	✓ +
Phylogenetics	✓++	✓ +++	×
Protein Translation View	✓	✓	✓
Primer Design and Analysis	×	✓	×
Bioinformatic Databases:			
Genome Searching and Information	×	✓ +++	✓ ++
Text and Literature Searching	×	✓	×
API (Plug-in development kit)	×	✓	×
Layout	++	+++	+
Overall Ease of Use	+++	++	++
System Compatibility (minimum)	Windows 2000, 512 MB RAM and 500MHz Pentium 3 Processor; Mac OS X V ersion 10.4, 512 MB RAM and 400 MHz processor	Windows XP 2GB RAM, 64-bit PS and 500 MHz, Pentium3; Mac OS X version 10.4, 2 GB RAM, 64-bit OS, 500 MHz	Windows 2000, 512 MB RAM, 500 MHz, Pentium 3; Mac OS X version 10.4, 512 MB, 400 MHz, G3
Licence Cost	$1440	Ranges from $395 - $3995 depending academic / commercial purposes and single / multiple users	Cost varies with features requested

The following table describes the symbols that have been used in feature comparison.

Key to table 1

✓ A check mark is awarded if the feature is available in the program

× A cross is given if the feature is not present in the program
If the feature is available, it is scored with '+' marks when applicable, explained below.

\+ One plus sign is given if the feature is at a basic level, hard to use, or lacks accuracy

++ Two plus signs are given if the feature is accurate

+++ Three plus signs are given if the feature is accurate, intuitive, and easy to use

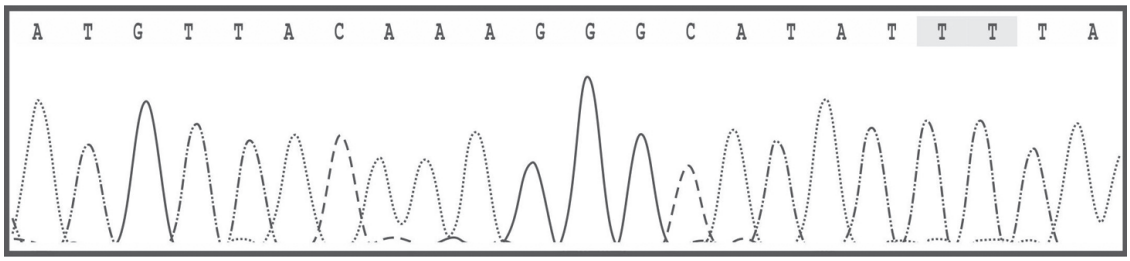

Fig. 3: Electropherogarm with no interference. All four nucleotide peaks are represented as follows: A (·····), G (—), C (– –) and T (– · · –).

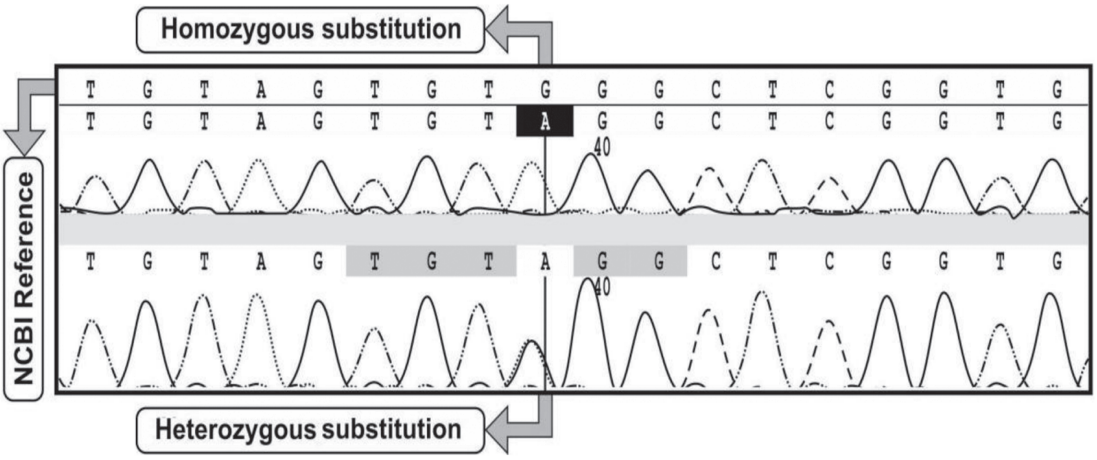

Fig. 4: Electropherogram showing homozygous and heterozygous substitutions. All four nucleotide peaks are represented as follows: A (·····), G (—), C (– –) and T (– · · –).

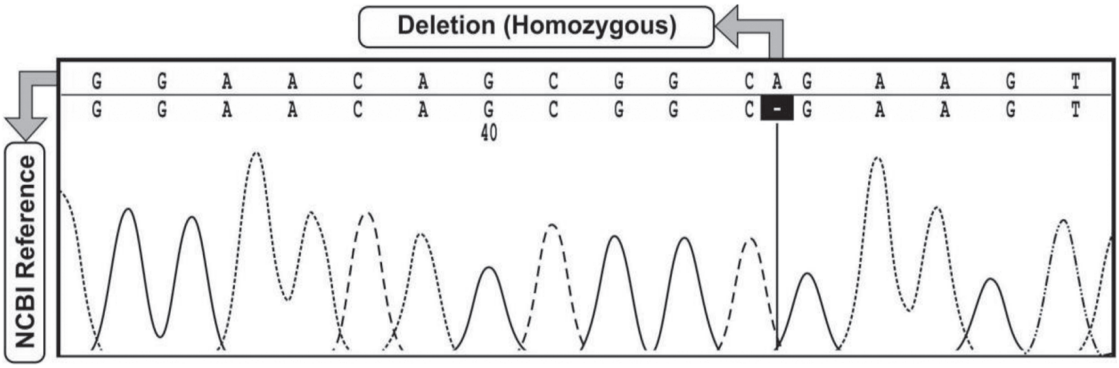

Fig. 5: Electropherogram showing homozygous deletions. All four nucleotide peaks are represented as follows: A (·····), G (—), C (– –) and T (– · · –).

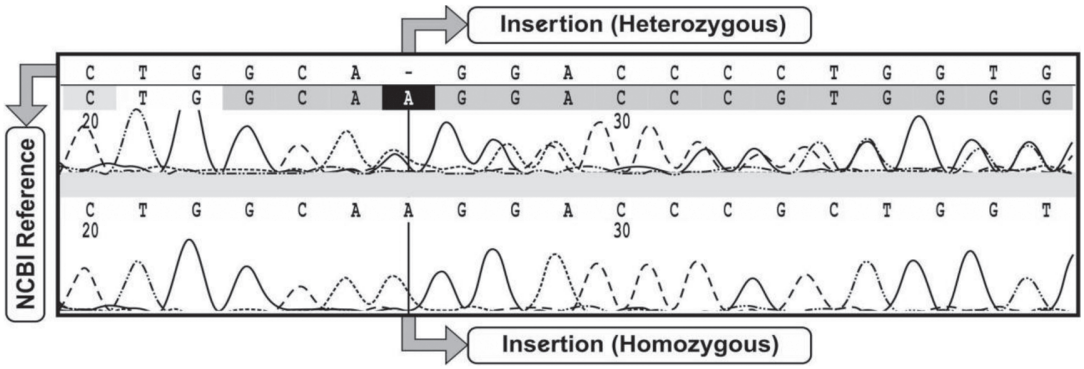

Fig. 6: Electropherogram showing homozygous and heterozygous insertion. All four nucleotide peaks are represented as follows: A (·····), G (—), C (– –) and T (– · · –).

between one and three + symbols are added to indicate accuracy and ease of use of a particular feature. One + symbol indicate that the feature is present at a very basic level, or is not intuitive. Two + symbols show that the feature is completely accurate, and is adequate for the user of the program. Three + symbols indicate that the feature is completely accurate, easy to use, and is efficient or saves time. This table below has been made in an attempt to display the various features of these three software programs, in order to help the reader in determining which program is best suited for his/her use.

The layout of sequencing analysis for representing various mutations: homo and heterozygous substitutions (Fig. 4), homo deletions (Fig. 5) and homo and heterozygous insertions (Fig. 6) is shown as an example from software CodonCode Aligner. The following features are invariably present in all three software programs: chromatogram editing and assembly, reference mapping and alignment, variant detection, and SNP finder and analysis. The other features which are only present in some programs will be further discussed.

Internal database creation and management is an option which allows personal files and projects to be saved within the program, which open as a sidebar each time the program is opened. This feature is present only in Geneious. This feature is absent in the other two software programs, where each project must be saved individually.

SNP discovery is an automatic feature important for understanding genetic variations, and it also speeds up the process of finding variants between different patient samples, or between a patient sample and a reference sequence. While there is a 'find mutations' option in CodonCode Aligner, it does not help the process of specifically finding SNPs – it simply points out, at a glance, the variants within a given set of samples. Geneious, however, contains a more complex version of this feature.

Variant detection involves the recognition of substitutions, duplications, indels, SNPs, and mutations for manual checking. In commercial applications of genomics, it is crucial that variant detection can be executed quickly and efficiently, as users of these software programs often have thousands of DNA sequences to cross check. All three programs contain this feature. CodonCode Aligner highlights bases that do not match the corresponding base in the reference sequence, in red so that these mismatching bases are easily identified. Geneious too does this but each base is highlighted in a different

color. Sequencher indicates mismatches by underlining the base. Therefore, in those programs where mismatches are highlighted in different colors, it is easier to detect variants.

Reference sequence information searching includes, but is not limited to, full genomes of organisms, specific protein sequences, and BLAST searches (6). This exists as an interface connection to Bioinformatics databases including NCBI, UniProt and others. While, this connection is available with Geneious program, it is not available with CodonCode Aligner and Sequencher programs. In addition to a discussion of the salient features of these three programs, user-friendliness will now be discussed. The overall layout is important to discuss as it adds to or detracts from the overall ease of use. We found Geneious layout very helpful because it contains three windows: a sidebar of all available folders, projects, and databases, a top window with a list of all the samples in the folder being currently viewed, and a bottom window showing the sequences. Sequencher has basic layout requiring some time to adjust. The layout of CodonCode Aligner is slightly better, but it is not as intuitive as that of Geneious.

Conclusion

Over the past few years, DNA Sanger sequencing technology has evolved from a rare occurrence to a commercial and scientific commodity. An increasing number of software programs are available for DNA sequencing analysis with more or less similar features, but with few excelling features in unique. As DNA sequencing technology continues to evolve and the needs of scientists continue to grow variably, these programs will become more specific and will tend to become tailored to individual needs.

The findings and conclusions in this report are those of the authors and do not necessarily represent the potentials of the software. Names of vendors or manufacturers are provided as examples of available sources; inclusion does not imply endorsement of the vendors, manufacturers.

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References

1. Tsiatis, A.C., Norris-Kirby, A., Rich, R.G., Hafez, M.J., Gocke, C.D., Eshleman, J.R. and Murphy, K.M. (2010). Comparison of Sanger Sequencing, Pyrosequencing, and Melting Curve Analysis for the Detection of *KRAS* Mutations. J Mol Diagn, 12(4):425-32.
2. CodonCode Corporation: http://www.codoncode.com/aligner/.
3. Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P. and Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, 28(12):1647-1649.
4. Nishimura, D. (2000). Sequencher 3.1.1. Biotech Software & Internet Report, 1(1-2):24-30.
5. Gilbert, D. (2004). Bioinformatics software resources. Briefings in Bioinformatics, 5(3):300-304.
6. Ramakrishna, N. (2002). The emerging landscape of bioinformatics software systems. Computer, 35(7):41-45.

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High Fat Diet Influences Spatial Learning and Brain Cholinergic and Antioxidant Systems in Male Rats

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Abstract

Diets rich in high fats have shown to exert detrimental effects on brain chemistry and cognitive functions. The present study was designed to examine the high fat (HF) diet induced age related perturbations in cholinergic and antioxidant systems of brain and associated dysfunctions in cognitive behavior of rats. Wistar albino rats (2 and 6 months) were fed with control and HF diet separately for a period of 8 weeks. It is evident that no significant changes were observed in body weight except in 7th and 8th week after consumption of HF diet. However, significant increased levels in total cholesterol and triglycerides were recorded in both 4 and 8 months age groups of HF diet fed rats. Results also showed decreased synaptosomal acetylcholinesterase (AChE) and, mitochondrial superoxide dismutase (SOD) activities where as MDA levels increased in the cortex, hippocampus and cerebellum regions of both age groups following consumption of HF diet. These HF diet induced alterations in cholinergic and antioxidant systems were greater in cortex and more pronounced at 8 months age rats. Spatial learning, memory and exploratory behaviors also decreased in 4 and 8months age groups of HF diet fed rats. In conclusion, our results suggest that HF diet-induces impairments in spatial learning and alterations in brain cholinergic and antioxidant systems in age dependent manner.

Keywords: high fat diet, cholinergic system, antioxidant system, behavior, brain.

Introduction

The consumption of fat-rich diets has increased significantly over the past decade and contributing to the world wide epidemic obesity (1). The high-fat (HF) diet consumption has been linked to development of chronic diseases including diabetes, hypertension, cardiovascular diseases and also cognitive function deficits (2-4). Epidemiological studies have established an association between high intake of fats and increased risk of developing cognitive impairment (5,6). Different experimental studies also showed that consumption of HF diet is associated with increased vulnerability of cholinergic system and deficits in cognitive functions (7,8).

Recent studies showed altered brain cholinergic system and deficits in memory and learning, exploratory behavior after consumption of HF diets (9,10). Experimental and epidemiological studies have also showed that obesity and metabolic dysfunctions are highly associated with increased oxidative stress (11,12). Rinder *et al*., (13) and Dhibi *et al*., (14) reported that HF diet increased the lipid peroxidation and alterations in the markers of apoptosis by impairment in the activities of antioxidant enzymes superoxide dismutase, (SOD) catalase (CAT) and glutathione peroxidase (GPx) in the tissues of rat. Most of the available research studies on the impact of HF diet concentrated on old age rats and less attention has been focused on age-related impairments in cognitive functions. Therefore,

this study was undertaken to determine the HF diet induced age related impairments in brain cholinergic and antioxidant systems and associated dysfunctions in spatial learning and exploratory behavior of rats.

Materials and Methods

For the present study, 2 months and 6 months old male Wistar albino rats were fed with a control and high fat diets separately for a period of 8 weeks.

***Diet*:** The animals were fed in the laboratory with high fat and control diets supplied by National Institute of Nutrition (NIN), Hyderabad, India and water *ad libitum.* The High fat diet contained the following ingredients: Casein-2.736 Kg, L-cystine-0.024 Kg, Starch - 1.376 Kg, Sucrose - 1.376 Kg, Cellulose-0.40 Kg, Ground Nut oil-0.200 Kg, Tallow- 1.52 Kg, Mineral Mix (AIN 93 grade) - 0.28 Kg, Vitamin Mix (AIN 93 grade) - 0.08 Kg. The animals were housed in clear plastic cages with hardwood bedding in a room maintained at $28^{o} \pm 2^{o}$ C and relative humidity 60 ± 10% with a 12 hour day/night cycle. Animal experiments were carried out in accordance with NIH and ICMR (India) guidelines. The approval to conduct the experiments was obtained from the Institutional animal ethical clearance committee, S. V. University. Both age groups of rats were weighed every week for the period of two months.

***Serum total cholesterol and triglycerides*:** Total cholesterol and triglycerides were estimated by using commercially available kits (bio systems) with auto analyzer in serum of control and high fat diet fed rats at 4 and 8 months.

***Isolation of mitochondrial and synaptosomal fractions*:** Synaptosomes were isolated from brain homogenates using Ficoll-sucrose gradients (15). The cerebral cortex, cerebellum and hippocampus were isolated in cold conditions. The tissues were weighed and homogenized in 10 ml of ice-cold homogenizing buffer (320 mM sucrose, 10 mM imidazole, 1 mM EDTA) and the volume was brought up to 25 ml with homogenizing buffer. The homogenates were centrifuged at 750 × g for 10 min. The pellets were discarded. The supernatants were centrifuged at 17,000 × g for 20 min. The pellets were suspended in 10 ml of 0.32 M sucrose and were layered on a two-step discontinuous Ficoll–sucrose gradient consisting 13 and 7.5% Ficoll and centrifuged at 65,000 × g for 45 min. The milky layer was formed at the interface of 13 and 7.5% Ficoll. The pellet formed at the bottom of the centrifuge tube (mitochondrial fraction) was taken and suspended in homogenizing buffer. The milky layer fraction was diluted with nine volumes of 0.32 M sucrose and centrifuged again 17,000 × g for 30 min. The supernatant was discarded and the pellet (synaptosomal fraction) was suspended in 0.32 M sucrose.

Cholinergic system

Estimation of Acetylcholine (ACh): The acetylcholine content was estimated by the method of Metcalf (16) as given by Augustinson (17). The synaptosomal fractions of hippocampus and cerebellum were placed in boiling water for 5 minutes to terminate the AChE activity and also to release the bound ACh. To the synaptosomal fractions 1 ml of alkaline hydroxylamine hydrochloride followed by 1 ml of 50% hydrochloric acid were added. The contents were mixed thoroughly and centrifuged. To the supernatant 0.5 ml 0.37 M ferric chloride solution was added and the brown colour developed was read at 540 nm against a reagent blank in a spectrophotometer and expressed as acetylcholine (ACh) content (µ moles of ACh/gm wt.).

Estimation of Acetylcholinesterase activity (AChE): AChE specific activity was determined following the method of Ellman *et al.*,(18). The reaction mixture contained 3.0 ml of phosphate buffer (pH 8.0), 20 µl of 0.075M acetylthiocholine iodide (substrate) and 100 µl of 0.01 M DTNB (5, 5-Dithiobis-2-Nitrobenzoic acid). The reaction was initiated with the addition of 100 µl of synaptosomal fraction. The contents were incubated for 30 min at room temperature and the color absorbance was measured at 412 nm in spectrophotometer.

Oxidative stress marker enzymes

Estimation of Superoxide dismutase (SOD) activity: Measurement of total SOD activity was performed according to Misra and Fridovich (19) based on the inhibition of autoxidation of epinephrine. The total reaction mixture contained of 880 µ l of carbonate buffer, 20 µl of epinephrine and 100 µl of enzyme source and absorbance was recorded at 480 nm against reagent blank. The enzyme activity was expressed as µ moles of superoxide anion reduced/ mg of protein.

Estimation of MDA levels: The lipid peroxides were determined by the TBA method of Hiroshi *et al*.,(20). The tissues were homogenized in 1.15% KCl (20% W/V). To 1 ml of tissue homogenate, 2.5 ml of 20% trichloro acetic acid (TCA) was added and the contents were centrifuged at 3500g for 10 minutes. Residue was dissolved in 2.5 ml of 0.05 M sulphuric acid (H2SO4). To this 3 ml of thiobarbituric acid (TBA) was added and the samples were kept in a hot water bath for 30 minutes. The samples were cooled and malondialdehyde (MDA) was extracted with 4 ml of n-butanol and read at 530 nm in a spectrophotometer against the reagent blank. The results were expressed as micromoles of MDA formed/mg of tissue/hr.

Estimation of protein content: Protein content of the tissues was estimated by the method of Lowry *et al*., (21). To 0.1 ml of synaptosomal fraction, 1ml 10%TCA was added and the samples were centrifuged at 1000g for 15 minutes. Supernatant was discarded and the residues were dissolved in 1ml of 1N sodium hydroxide (NaOH). To this, 2 ml of alkaline copper reagent was added followed by 0.2 ml of folin-phenol reagent (1:1folin:H2O). The color was measured at 600 nm in spectrophotometer (Hitachi model U-2000) against a blank. The protein content in mitochondrial fraction was also measured with the same procedure. The protein content of the tissues was calculated using the standard graph. The values were expressed as mg protein/gm wet weight of the tissue.

Behavioral Studies

Morris Water Maze: The water maze is a circular water tank measuring 1.85 m in diameter and 0.7 m deep constructed according to a basic design similar to that of Morris (22). Four points along the circumference of the water tank are designated arbitrarily North (N), South (S), East (E) and West (W), thus dividing the maze into four quadrants. The pool was filled to a depth of 30 cm with water made opaque with white, non-toxic water-based paint. A circular platform (diameter 12.5 cm) is placed 2-3 cm below the surface of the water. Rats were allowed to swim to the hidden platform and the escape latency (time to find the hidden platform) was noted. Animals were tested for a total of 2 daily trials for 5 days. Each trial was separated by 30 minutes. Rats were placed in water facing the wall of the pool and allowed 60 seconds to find the escape platform. If the animal reached the escape platform within 60 seconds, the escape latency was recorded and the mouse was allowed to rest on the platform for 10 seconds before removal. Each age group was separately tested. In tests of reference memory, the hidden platform remained in the same location throughout each phase of the experiment. During the acquisition phase, the platform was placed in the centre of the quadrant, 15 cm from the edge of the pool, and it was moved to the opposite quadrant for the reversal phase. In tests of working memory, each rat was required to find the hidden platform located in a new position. The location of the hidden platform was changed daily in a pseudo-randomized fashion such that different rats were tested in all quadrants on a given day, and all rats were trained in each quadrant at least twice during the experiment.

Exploratory behavior: Exploratory behavior was measured in a box with a hole board bottom (90 cm × 90 cm) containing three equally spaced holes (3 cm in diameter) in the floor. Each rat was placed in the center of the arena for 5 min during which time the number of head dips and head-dipping duration (in seconds) were

recorded. A head dip was scored if both eyes disappeared into the hole.

***Analysis of Data*:** The data were subjected to one way analysis of variance (ANOVA) followed by student Newman-Keuls (SNK) post hoc test. The 0.05 level of probability was used as the criterion for significance.

Results

In the present study, analysis of body weight over the 8 experimental weeks, established no significant changes (P >0.05) in body weight except in 7th and 8th week of 4 and 8 months age groups of HF diet fed rats, compared to controls (Fig.1). Total cholesterol and triglycerides levels in serum were estimated using Bio-system kits. A significant increase was observed in both total cholesterol and triglyceride levels in both the age groups of HF diet fed rats compared to controls (Fig. 2 and 3). The specific activity of synaptosomal AChE and ACh levels were estimated in cortex, cerebellum and hippocampus regions at 4 and 8 months age groups of rats (Fig. 4 and 5). The hippocampus region exhibited higher levels of AChE activity and ACh levels than cortex and cerebellum (Fig. 4 and 5). The specific activity of AChE as well as ACh levels were decreased in HF diet fed rats (The decrease in AChE was 3.9% and 21.1% in cortex, 2.3% and 16% in cerebellum, and 9.4% and 29.1% in hippocampus regions respectively of 4 and 8 months age groups of rats where as the decrease in ACh levels were 25.3% and, 33% in cortex, 13.3% and 31% in cerebellum and 9.2% and 21.1% in hippocampus regions respectively of 4 and 8 months age groups of rats) compared to controls. (Fig. 4 and 5).

Further, we evaluated the mitochondrial SOD activity and tissue MDA levels in cortex, cerebellum and hippocampus regions (Fig. 6 and 7). The SOD enzyme activity and MDA levels were increased with age and the highest activity of SOD and MDA levels were observed in cortex than cerebellum and hippocampus. HF diet fed rats showed decrease in the activity of SOD (cortex: 9.7% in 4 months, 32% in 8 months; cerebellum: 5.4% in 4 months, 20.1% in 8

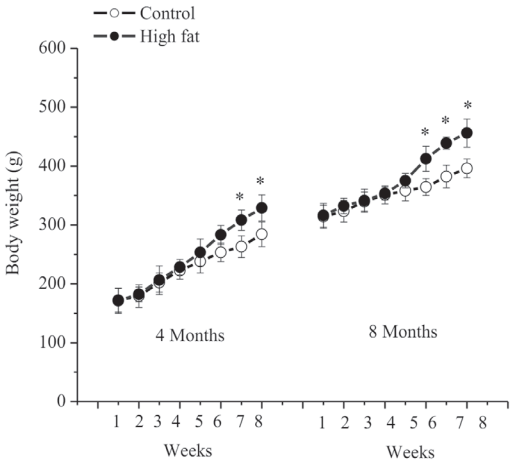

Fig. 1. Effect of HF diet on body weight of 4 and 8 months age groups of rats. Male rats were fed with control and high fat diets separately for a period of 8 weeks. Values are mean ± SD of eight observations. The values marked with ‘‘asterisk’’ are significantly different from corresponding controls as evaluated by the ANOVA followed by student Newman– Keuls (SNK) post hoc test (P < 0.05).

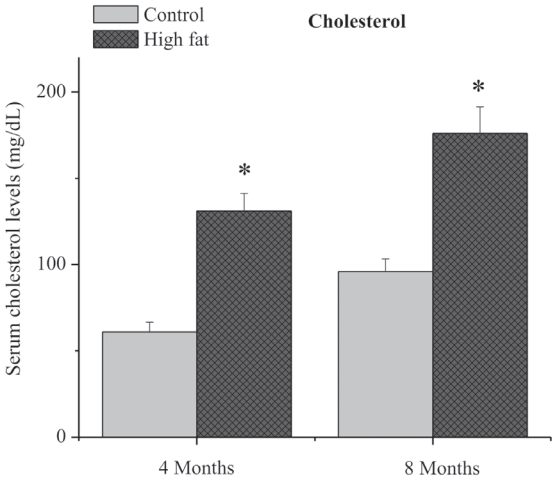

Fig. 2. Effect of HF diet on serum cholesterol levels in 4 and 8 months age groups of rats. Male rats were fed with control and high fat diets separately for a period of 8 weeks. Values are mean ± SD of eight observations. The values marked with ‘‘asterisk’’ are significantly different from corresponding controls as evaluated by the ANOVA followed by student Newman– Keuls (SNK) post hoc test (P < 0.05).

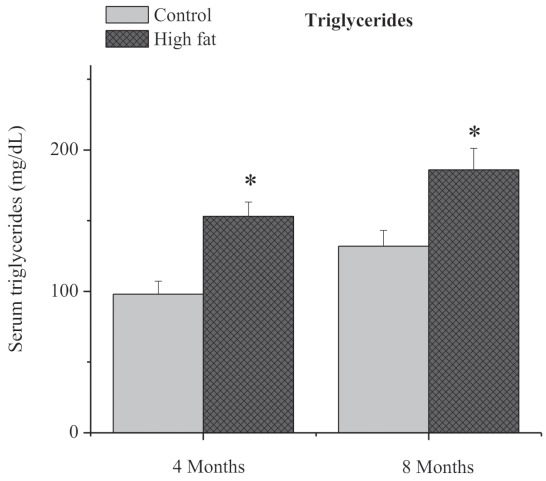

Fig.3. Effect of HF diet on serum triglyceride levels in 4 and 8 months age groups of rats. Male rats were fed with control and high fat diets separately for a period of 8 weeks. Values are mean ± SD of eight observations. The values marked with ''asterisk'' are significantly different from corresponding controls as evaluated by the ANOVA followed by student Newman– Keuls (SNK) post hoc test ($P < 0.05$).

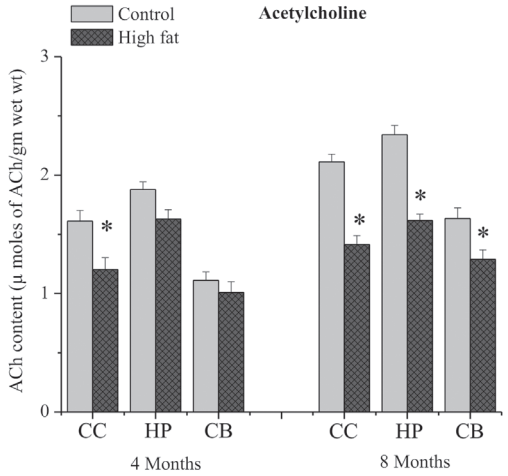

Fig.5. Effect of HF diet on synaptosomal acetylcholine (ACh) levels in cortex (CC), cerebellum (CB) and hippocampus (HP) regions of 4 and 8 months age groups of rats. Male rats were fed with control and high fat diets separately for a period of 8 weeks. Values are mean ± SD of eight observations. The values marked with ''asterisk'' are significantly different from corresponding controls as evaluated by the ANOVA followed by student Newman– Keuls (SNK) post hoc test ($P < 0.05$).

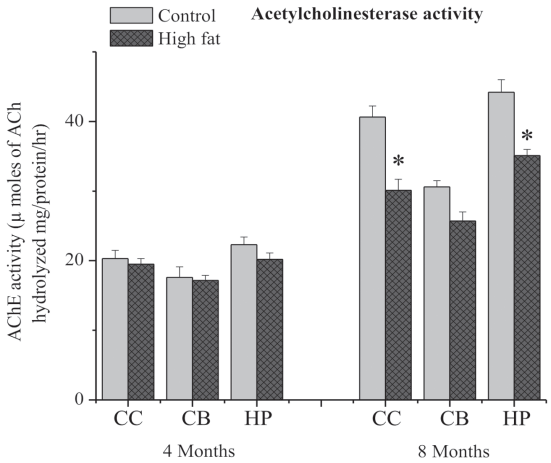

Fig.4. Effect of HF diet on synaptosomal acetylcholinestrase (AChE) activity in cortex, cerebellum and hippocampus regions of 4 and 8 months age groups of rats. Male rats were fed with control and high fat diets separately for a period of 8 weeks. Values are mean ± SD of eight observations. The values marked with ''asterisk'' are significantly different from corresponding controls as evaluated by the ANOVA followed by student Newman– Keuls (SNK) post hoc test ($P < 0.05$).

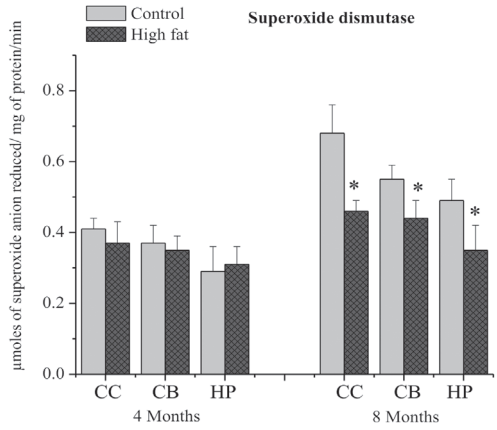

Fig.6. Effect of HF diet on mitochondrial superoxide dismutase activity (SOD) in cortex (CC), cerebellum (CB) and hippocampus (HP) regions of 4 and 8 months age groups of rats. Male rats were fed with control and high fat diets separately for a period of 8 weeks. Values are mean ± SD of eight observations. The values marked with ''asterisk'' are significantly different from corresponding controls as evaluated by the ANOVA followed by student Newman– Keuls (SNK) post hoc test ($P < 0.05$).

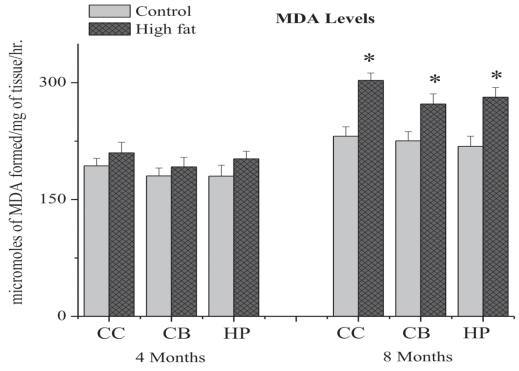

Fig.7. Effect of HF diet on MDA levels in cortex (CC), cerebellum (CB) and hippocampus (HP) regions of 4 and 8 months age groups of rats. Male rats were fed with control and high fat diets separately for a period of 8 weeks. Values are mean ± SD of eight observations. The values marked with ''asterisk'' are significantly different from corresponding controls as evaluated by the ANOVA followed by student Newman– Keuls (SNK) post hoc test ($P < 0.05$).

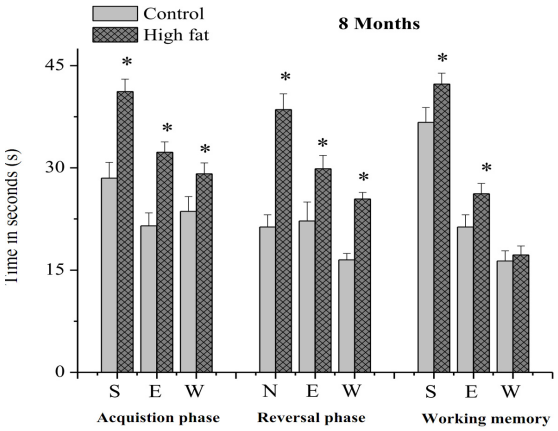

Fig.9. Effect of HF diet on average escape latency (time in sec) to find the hidden platform during acquisition, reversal phase tests and working memory test at 8 months age group rats. Values are mean ± SD of eight observations. The values marked with ''asterisk'' are significantly different from corresponding controls as evaluated by the ANOVA followed by student Newman– Keuls (SNK) post hoc test ($P < 0.05$).

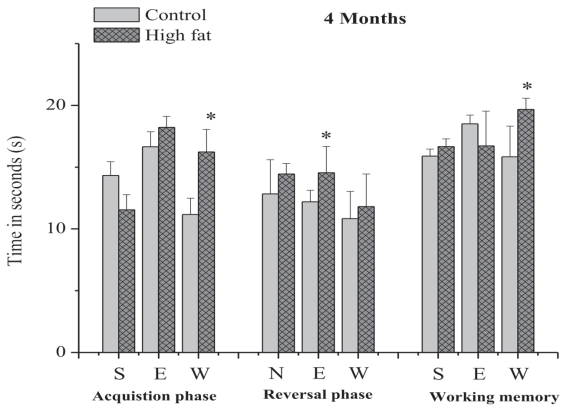

Fig.8. Effect of HF diet on average escape latency (time in sec) to find the hidden platform during acquisition, reversal phase tests and working memory test at 4 months age group rats. Values are mean ± SD of eight observations. The values marked with ''asterisk'' are significantly different from corresponding controls as evaluated by the ANOVA followed by student Newman– Keuls (SNK) post hoc test ($P < 0.05$).

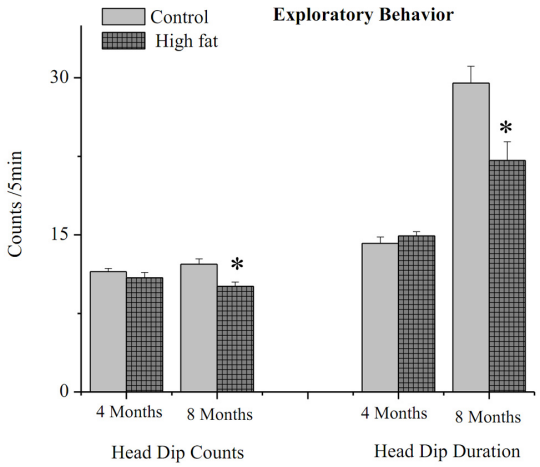

Fig.10. Effect of HF diet on exploratory behavior at 4 and 8 months age groups of rats. Values are mean ± SD of eight observations. The values marked with ''asterisk'' are significantly different from corresponding controls as evaluated by the ANOVA followed by student Newman– Keuls (SNK) post hoc test ($P < 0.05$).

months; hippocampus: 6.8% in 4 months, 28.5% in 8 months) and increase in tissue MDA levels (cortex: 8.6% in 4 months, 30.9% in 8 months; cerebellum: 6.4% in 4 months, 20.9% in 8 months; hippocampus: 12.2% in 4 months, 28.9% in 8 months) in both age groups of rats, compared to controls (Fig. 6 and 7). However, the alterations in specific enzyme activities, neurotransmitter and MDA levels were more pronounced in cortex ($P < 0.001$) than hippocampus and cerebellum regions and greater at 8 months ($P < 0.001$) age groups of rats (Fig.4 to 7) after consumption of HF diet.

Spatial learning and memory were tested in Morris water maze and results from the reference (acquisition and reversal phases) and working memory tests are summarized in Fig. 8 and 9. HF diet fed rats showed increase in the escape latency to find the hidden plat form in acquisition, reversal phases and working memory of both age groups of rats, compared to control (Fig.8 and 9). The exploratory behavior recorded in three whole board showed shorter head dip duration and lesser head dip counts in HF diet fed rats of both age groups of rats (Fig.10). However, the alterations observed in spatial learning, memory and exploratory behavior in older adults were significant ($P < 0.001$) (Fig. 8 to 10) and marginal ($P > 0.05$) in young adult rats.

Discussion

The major findings of the present study are that, consumption of HF diet can lead to impairments in spatial learning, memory and brain cholinergic and antioxidant systems in different age groups of rats. HF diet induced an overt obesity characterized by body weight gain and fat deposition in different tissues (1,23). Rats fed with the HF diet had consistently greater body weight than control and the majority of the weight gain occurred from the fourth week in both age groups of rats after consumption of HF diet. Furthermore, the increase in body weight was greater in older adults (8 months) compared to young adults after consumption of HF diet. Chen *et al.,*(24) reported similar body weight gain after six weeks in HF diet fed rats.

Diets rich in HF induce alterations in lipid profiles which consequently increase the serum total cholesterol and triglyceride levels in rats (25). Our results also showed significant increase in serum total cholesterol and triglyceride levels following consumption of HF diet in both age groups of rats. Roberst *et al*., (26) reported that HF diet caused an increase in serum triglycerides and total cholesterol levels of rats. Different experimental studies also showed increased levels of lipid profiles in serum and tissues of rats fed with HF diets (27,28). Interestingly, the present study results showed HF diet induced alterations in serum lipid profiles were greater in older rats than young adults, suggesting age dependent accommodation to the higher caloric density of the HF diet. The link between dyslipidemia and risk of cognitive impairment or dementia is unclear (29). Indeed, rats fed with higher amount of dietary fat showed widespread cognitive deficits on various tasks of learning and memory (10,30). The results of present study showed HF diet fed rats took longer time to find hidden platform and caused impairments in acquisition, reversal phases and working memory in Morris water maze test at 4 and 8 months age groups of rats. Molteni *et al.,*(31) reported that HF diet consumption increases the deficits in spatial learning and memory of rats. Impairments in learning and memory have also been observed in young, adult and aged rats after consumption of HF diet (10,32). Our findings also showed decreased exploratory behavior in both age groups of rats following HF diet consumption. However, HF diet induced impairments in spatial learning, memory and exploratory behavior were greater in older age group rats compared to young adult rats suggesting age dependent effects of HF diet. However, the mechanisms of effects of dietary fat on cognitive functions are still not completely understood.

Cognitive dysfunctions are characterized by a substantial loss of cholinergic system in different brain regions of rat (6,33). Morganstern *et al*.,(10) reported that changes in cognitive and exploratory behaviors induced by consumption

of HF diet may stem from disturbances in cholinergic system in specific brain regions. Another study also reported that HF diet consumption disturb the brain cholinergic system in rats (34). However, most of the studies were concentrated on whole brain evaluation and missed the presence of brain region specific elevations in response to HF diet consumption (5,7,35). In this connection, present study evaluated the brain region specific alterations in cholinergic system (ACh and AChE) in two different age groups of rats. Our present findings demonstrated that synaptosomal ACh levels and AChE enzyme activity decreased following consumption of HF diet in cortex, hippocampus and cerebellum regions at 4 and 8 months age groups of rats in an age dependent manner. Kaizer *et al*.,(36) also reported that AChE activity and ACh levels were significantly decreased in cortex, hippocampus and hypothalamus regions following consumption of HF diet. Additionally *in vitro* studies have also suggested that fat intake decreases the AChE activity and ACh levels (37-39). It is also proved in another study, which showed that 8 weeks of HF diet consumption depletes the brain ACh levels by modifying the AChE enzyme activity (9,10). However, the decreased activity of AChE enzyme and levels of ACh in different brain regions indicate that HF diet influences the cholinergic system in brain region specific manner and contributing to the observed changes in spatial learning, memory and exploratory behaviors of rats.

The mechanisms by which HF diet induce alterations in cholinergic system are not clearly known (40). HF diets increase oxidation of fatty acids through the peroxisomal oxidation pathway, that is associated with increased generation of free radicals and reduce the antioxidant enzyme activities (11,41). The present study confirms that HF diet consumption caused decrease in mitochondrial SOD enzyme activity where as MDA levels increased in cortex, cerebellum and hippocampus regions at both age groups of rats. Several recent studies also suggested that HF diet fed rats showed decrease in the antioxidant enzyme activities and increase in the MDA levels in brain of rats (13,42,43). HF diet caused oxidative damage in brain by enhancing peroxidation of membrane lipids due to the generation of ROS and decreases the activity levels of antioxidant enzymes leading to oxidative stress (12,43). Our results clearly showed that an increased MDA level has been accompanied by reduction in the activity of SOD enzyme in different brain regions of rat. The decreased antioxidant enzymes activities in HF diet fed rats are indicative of the oxidative stress and a response to the cholinergic system dysfunction.

We have chosen to evaluate brain regions instead of whole brain because different regions may respond differently to oxidative stress and vulnerability of the cholinergic system (35,40). In the present study, the alterations were more pronounced in cortex compared to hippocampus and cerebellum regions. Previous studies also suggested that cortex region is more susceptible to HF diet consumption (6,35). However, HF diet induced impairments in cognitive functions and brain chemistry appears to be age specific (10,44,45). In our study, HF diet induced alterations in cognitive functions and brain cholinergic and antioxidant systems were marginal in young adults compared to older adults, perhaps longer HF diet consumption is required to alter cognitive functions in young adult rats. In conclusion, the present data indicate that HF diet influences spatial learning, memory and exploratory behavior and associated cholinergic and antioxidant systems in a brain region specific manner and suggest that age is a major factor in determining the detrimental effects of high fat diet.

Acknowledgements

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References

1. Taraschenko, O.D., Maisonneuve, I.M. and Glick,S.D.(2011). Sex differences in high fat-induced obesity in rats: Effects of 18-methoxycoronaridine. Physiol Behav, 103(3-4):308-14.

2. Olufadi, R. and Byrne, C.D. (2008). Clinical and laboratory diagnosis of the metabolic syndrome. J Clin Pathol, 61(6):697-706.

3. Park, E.J., Lee, J.H., Yu, G.Y., He, G., Ali, S.R., Holzer, R.G., Osterreicher, C.H., Takahashi, H. and Karin, M. (2010). Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. Cell, 22;140 (2):197-208.

4. Lu, J., Wu, DM., Zheng, Y.L., Hu, B., Cheng, W., Zhang, Z.F. and Shan, Q. (2011). Ursolic acid improves high fat diet-induced cognitive impairments by blocking endoplasmic reticulum stress and IκB kinase α/nuclear factor-κB-mediated inflammatory pathways in mice. Brain Behav Immun, 25(8):1658-67.

5. Francis, H. and Stevenson, R. (2013). The longer- term impacts of Western diet on human cognition and the brain. Appetite, 63:119-28.

6. Ullrich, C., Pirchl, M. and Humpel, C. (2010). Hypercholestero lemia in rats impairs the cholinergic system and leads to memory deficits. Mol Cell Neurosci, 45(4):408-17.

7. Lindqvist, A., Mohapel, P., Bouter, B., Frielingsdorf, H., Pizzo, D., Brundin, and P., Erlanson-Albertsson, C. (2006). High-fat diet impairs hippocampal neurogenesis in male rats. Eur J Neurol, 13(12):1385-8.

8. Granholm, A.C., Bimonte-Nelson, H.A., Moore, A.B., Nelson, M.E., Freeman, L.R. and Sambamurti, K. (2008). Effects of a saturated fat and high cholesterol diet on memory and hippocampal morphology in the middle-aged rat. J Alzheimers Dis, 14(2):133-45.

9. Kosari, S., Badoer, E., Nguyen, J.C., Killcross, A.S. and Jenkins, T.A. (2012). Effect of western and high fat diets on memory and cholinergic measures in the rat. Behav Brain Res, 1; 235(1):98-103.

10. Morganstern, I., Ye, Z., Liang, S., Fagan, S. and Leibowitz, SF. (2012). Involvement of cholinergic mechanisms in the behavioral effects of dietary fat consumption. Brain Res, 27; 1470:24-34.

11. Stranahan, A.M., Cutler, R.G., Button, C., Telljohann, R. and Mattson, M.P. (2011). Dietnduced elevations in serum cholesterol are associated with alterations in hippocampal lipid metabolism and increased oxidative stress. J Neurochem, 118(4):611-5.

12. Charradi, K., Elkahoui, S., Limam, F. and Aouani, E. (2013). High fat diet induced an oxidative stress in white adipose tissue and disturbed plasma transition metals in rat: prevention by grape seed and skin extract. J Physiol Sci, 63(6):445-55.

13. Rindler PM, Plafker SM, Szweda LI, and Kinter M. High dietary fat selectively increases catalase expression within cardiac mitochondria. J Biol Chem. 2013 Jan 18; 288(3):1979-90.

14. Dhibi, M., Brahmi, F., Mnari, A., Houas, Z., Chargui, I., Bchir, L., Gazzah, N,, Alsaif MA, and Hammami M. (2011). The intake of high fat diet with different trans fatty acid levels differentially induces oxidative stress and non alcoholic fatty liver disease (NAFLD) in rats. Nutr Metab (Lond), 23;8(1):65.

15. Cotman, CW. and Matthews, D.A. (1971). Synaptic plasma membranes from rat brain synaptosomes:Isolation and partial characterization. Biochem Biophys Acta, 249, 380-394.

16. Metcalf W.K. (1951). A simplified technique of spectrography and its application to the study of intracellular hemoglobin. Blood, 6(11): 1114-22.

17. Augustinson, K.B. (1963), Cholinesterase and anticholinesterase agents. (Ed. G.B. Koelle). Springer Verlag Berlin, 5: 89-128.

18. Ellman, G.L, Courtney, K.D., Andres, V.R.M. and Feather stone, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol, 7: 88-95.

19. Misra, H.P. and Fridovich, I. (1972). The role of superoxide anion in the autooxidation of epinephrine and a simple assay for Superoxide dismutase. The Jour Biological Chem, 247: 3170-3175.

20. Hiroshi, O., Ohisi, N. and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by TBA reaction. Anal Biochem, 95:351-358.

21. Lowry, O,H., Rosenbrough, N.J., Farr, AL. and Randal, R.J. (1951). Protein measurement with Folin phenol reagent. J Biol Chem, 193: 265-275.

22. Morris, R.G.M. (1984). Developments of water maze procedure for studying spatial learning in the rat. J Neurosci Meth, 11: 47-60.

23. McDonald, R.B., Carlson, K., Day, C., Stern, J.S. and Horwitz, B.A. (1989). Effect of gender on the response to a high fat diet in aging Fischer 344 rats. J Nutr, 119(10):1472-7.

24. Chen, W.P., Ho, B.Y., Lee, C.L., Lee, C.H. and Pan, T.M. (2008). Red mold rice prevents the development of obesity, dyslipidemia and hyperinsulinemia induced by high-fat diet. Int J Obes, 32:1694-704.

25. Wojczynski, M.K., Glasser, S.P., Oberman, A., Kabagambe, E.K., Hopkins, P.N., Tsai, M.Y., Straka, R.J., Ordovas, J.M. and Arnett, D.K. (2011). High-fat meal effect on LDL, HDL, and VLDL particle size and number in the Genetics of Lipid-Lowering Drugs and Diet Network (GOLDN): an interventional study. Lipids Health Dis, 18;10:181

26. Roberts, C,K., Izadpanah, A., Angadi, S.S. and Barnard, R.J. (2013). Effects of an intensive short-term diet and exercise intervention: comparison between normal-weight and obese children. Am J Physiol Regul Integr Comp Physiol, 305 (5): R552-7.

27. Ciapaite, J., van den Broek, N.M., Te, Brinke, H., Nicolay, K., Jeneson, J.A., Houten, S.M. and Prompers, J.J. (2011). Differential effects of short- and long-term high-fat diet feeding on hepatic fatty acid metabolism in rats. Biochim Biophys Acta, 1811(7-8):441-51.

28. Linan, K., Niu, L., Kang, W. and Zhang, P. (2012). Effects of a high fat diet on long-chain fatty acids composition in rats serum and liver. Food research International, 283-286.

29. Bojanic, D.D., Tarr, P.T., Gale, G.D., Smith, D.J., Bok ,D., Chen, B., Nusinowitz, S., Lövgren-Sandblom, A., Björkhem, I. and Edwards, PA. (2010). Differential expression and function of ABCG1 and ABCG4 during development and aging. J Lipid Res, 51(1):169-81.

30. Crichton, G.E., Murphy, K.J. and Bryan J. (2010). Dairy intake and cognitive health in middle-aged South Australians. Asia Pac J Clin Nutr, 19(2):161-71.

31. Molteni, R., Barnard, R.J., Ying, Z., Roberts, C.K. and Gómez-Pinilla, F. (2002). A high-fat, refined sugar diet reduces hippocampal brain-derived neurotrophic factor, neuronal plasticity, and learning. Neuroscience, 112(4):803-14.

32. Pauli-Pott, U., Albayrak, O., Hebebrand, J. and Pott, W. (2010). Association between inhibitory control capacity and body weight in overweight and obese children and adolescents: dependence on age and inhibitory control component.Child Neuropsychol, 16(6):592-603.

33. Mesulam, M. (2004). The cholinergic lesion of Alzheimer's disease: pivotal

factor or side show? Learn Mem, 11(1): 43-9.

34. Mufson, E.J., Ginsberg, S.D., Ikonomovic, M.D. and DeKosky, S.T. (2003). Human cholinergic basal forebrain: chemoanatomy and neurologic dysfunction. J Chem Neuroanat, 26(4):233-42.

35. Amin, K.A., Kamel, H.H. and Abd Eltawab, M.A. (2011). The relation of high fat diet, metabolic disturbances and brain oxidative dysfunction: modulation by hydroxy citric acid. Lipids Health Dis, 14;10:74.

36. Kaizer, R.R., da Silva, A.C., Morsch, V.M., Correa, M.C. and Schetinger, M.R. (2004). Diet-induced changes in AChE activity after long-term exposure. Neurochem Res, 29(12):2251-5.

37. Pistell, P.J., Morrison, C.D., Gupta, S., Knight, A.G., Keller, J.N., Ingram, D.K. and Bruce-Keller, A.J. (2010). Cognitive impairment following high fat diet consumption is associated with brain inflammation. J Neuroimmunol, 26;219(1-2):25-32.

38. Vajreswari, A., Rupalatha, M. and Rao, P.S. (2002). Effect of altered dietary n-6-to-n-3 fatty acid ratio on erythrocyte lipid composition and membrane-bound enzymes. J Nutr Sci Vitaminol (Tokyo). 48(5):365-70.

39. Ahrén, B., Simonsson, E., Scheurink, A.J, Mulder, H., Myrsén, U. and Sundler, F. (1997). Dissociated insulinotropic sensitivity to glucose and carbachol in high-fat diet-induced insulin resistance in C57BL/6J mice. Metabolism, 46(1):97-106.

40. Buettner, R., Newgard, C.B., Rhodes, C.J. and O'Doherty, R.M. (2000). Correction of diet-induced hyperglycemia, hyperinsulinemia, and skeletal muscle insulinresistance by moderate hyperlep-tinemia. Am J Physiol Endocrinol Metab, 278(3):E563-9.

41. Xie, C., Lund E.G., Turley, S.D., Russell, D.W. and Dietschy, J.M. (2003). Quantitation of two pathways for cholesterol excretion from the brain in normal mice and mice with neurodegeneration. J Lipid Res, 44(9):1780-9.

42. Fachinetto, R., Burger, M.E., Wagner, C., Wondracek, D.C., Brito, V.B., Nogueira, C.W., Ferreira, J. and Rocha, J.B. (2005). High fat diet increases the incidence of orofacial dyskinesia and oxidative stress in specific brain regions of rats. Pharmacol Biochem Behav, 81(3):585-92.

43. Marczuk-Krynicka D., Hryniewiecki, T., Paluszak, J., Krauss, H. and Nowak, D. (2009). High Fat Content in Diets and Oxidative Stress in Livers of Non-Diabetic and Diabetic Rats. Polish J. of Environ. Stud, 249-253

44. Bruce-Keller, A.J., White, C.L., Gupta, S., Knight, A.G., Pistell, P.J., Ingram, D.K., Morrison, C.D. and Keller, J.N. (2010). NOX activity in brain aging: exacerbation by high fat diet.Free Radic Biol Med, 1;49(1):22-30.

45. Pancani, T., Anderson, K.L., Brewer, L.D., Kadish, I., DeMoll, C., Landfield, P.W., Blalock, E.M., Porter, N.M. and Thibault, O. (2013). Effect of high-fat diet on metabolic indices, cognition, and neuronal physiology in aging F344 rats. Neurobiol Aging, 34(8):1977-87.

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Mutagenic effect of microwave radiation on exopolysaccharide production in *Xanthomonas campestris*

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Abstract

The exopolysaccharide produced by the gram-negative bacterium *Xanthomonas campestris* (known as xanthan gum) is a product of commercial importance. In the present study *X. campestris* was subjected to two different microwave (MW) powers (90 W and 450 W) for different time durations, and its effect on growth and exopolysaccharide (EPS) production was studied for multiple generations of the xanthan overproducing mutants obtained after MW treatment. As the MW treatment was performed under temperature-controlled condition (by placing the inoculum vial in an ice containing beaker, while exposing to MW), where the temperature did not cross 25°C, whatever alterations were observed in growth and xanthan production seem largely to have resulted from non-thermal effects of MW radiation. As the trait of xanthan overproduction was transferred from one generation of MW treated organism to the next, the MW effect can be said to be mutagenic. However this MW induced mutation was not found to be stable over multiple generations. Both the xanthan overproducing mutants obtained by two different MW treatments reverted back to the parent phenotype, suggesting the reversible nature of the MW induced mutations. Identifying mutagenic frequencies of MW radiation can pave way for large scale screening programmes employing MW mutagenesis as a tool for strain improvement, and yield genetically stable overproducing mutants.

Keywords: Microwave mutagenesis; Xanthan gum; Exopolysaccharide; Microwave specific effects; Non-thermal effects.

Introduction

Xanthomonas campestris is a gram-negative bacterium belonging to γ subdivision of the *Proteobacteria*. It is well known for its pathogenicity to cruciferous plants, and also as a producer of the acid exopolysaccharide xanthan (xanthan gum). Xanthan is a heteropolysaccharide with a cellulose-like backbone and trisaccharide side-chains of one glucuronate and two mannose residues that are attached to every second glucose moiety of the β (1-4)- linked glucan main chain (1). Xanthan is of commercial importance owing to its applications as a thickening agent and emulsifier in the pharmaceutical, nutritional, and oil drilling industries. It is produced by fermentation, and production exceeds 86,000 tons per annum (2). Xanthan suspensions are particularly attractive for the frozen-food industry owing to their high freeze-thaw stability. It has also found application as an additive to fruit drinks to reduce tooth decay, and as a hydrophilic matrix carrier for pharmaceuticals. Xanthan has also been suggested as a good base for shampoos, and for suspending adhesive agents for wallpapers. Suspension-stabilizing property of xanthan makes it suitable for production of sharp prints from dyes (1).

Strain improvement through various mutagenesis strategies has been an integral part

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of any successful microbial fermentation process. Mutation has been a major factor involved in the several- fold increases obtained in the production of microbial metabolites. The ability to genetically modify a microbial culture to higher productivity has been the most notable factor in keeping the fermentation industry in its viable and healthy state (3). Strain improvement strategies based on use of mutagenic chemicals, viruses or transposons are being used at a decreasing frequency, due to complexity of these processes. Radiation mutagenesis is an attractive alternative owing to its convenience, safety, and improved mutagenicity results. However, industrial bacterial strains can exhibit intolerance to ultraviolet, X-ray and γ-ray radiation (4). Microwave (MW) radiation breeding can emerge as a clean, effective, and easily applicable tool for strain improvement. It is claimed to deliver a high rate of positive mutation, and the mutant strains thus generated can be sufficiently stable (5). MW mutagenesis can also avoid the problem of photo-reactivation, which often is observed with ultraviolet radiation based mutagenesis.

MWs are electromagnetic waves with frequency between 0.3-300 GHz, equivalent to wavelength range of 1m - 1mm. Several biological applications of MW have been developed (6) e.g., MW sterilization (7-9) microwave assisted extraction (MAE) (10-11), etc. MWs have been used for mutagenesis in plants (12) as well as microorganisms (4, 13). Thermal and non thermal effects of MW on microorganisms have been reported by many researchers (14-18). In the present study *X. campestris* was subjected to two different MW powers (90 W and 450 W) for different time durations, and its effect on growth and exopolysaccharide (EPS) production was studied for multiple generations of the MW treated parent strain.

Materials and Methods

Test organism: *Xanthomonas campestris* (MTCC 2286) was procured from Microbial Type Culture Collection, Chandigarh.

MW treatment: Bacterial suspension was prepared in sterile normal saline, from an active culture growing on nutrient agar, whose turbidity was adjusted to that of 0.5 McFarland standard. Test culture (5 mL) in sterile screw capped glass vials (15 mL, Merck) was exposed to MW radiation (90 W or 450 W; 2450 MHz) in a domestic MW oven (Electrolux® EM30EC90SS). MW treatment at 90 W was given for three different time durations viz. 2, 4, and 6 min, whereas MW treatment at 450 W was given for 3 min. Vials inside the MW oven were placed in an ice containing beaker, so as to avoid any thermal heating. Temperature of the microbial suspension after MW treatment at 90 W did not go beyond 15°C, and while using MW at 450 W, it did not go beyond 25°C. The whole MW treatment was performed in an air-conditioned room. Untreated inoculum was used as control. Before MW treatment all the inoculum vials were put in ice for 5 min to nullify any variations in initial temperature. Test organism was immediately (in less than 5 min) inoculated into TY broth (HiMedia, Mumbai) supplemented with calcium chloride (0.7 g/L) (19) following MW treatment. Incubation was made at room temperature under shaking condition (~180 rpm) for 72 h.

EPS quantification: Once the incubation was over, following estimation of growth by measuring OD at 625 nm, culture broth was subjected to centrifugation at 7500 rpm for 10 min, and the cell-free supernatant (CFS) was used for EPS quantification using the method described in Li et al. (2012) with some modification. Briefly, 40 mL of chilled acetone was added to 20 mL of CFS, and allowed to stand for 30 min. The EPS precipitated thus was separated by filtration through pre-weighed Whatman # 1 filter paper (Whatman International Ltd., England). Filter paper was dried at 60°C for 24 h, and weight of EPS on paper was calculated.

Screening for mutants: Following the MW treatment, the treated inoculum was streaked on nutrient agar plate, and incubated at 28°C till appearance of visible growth (which usually took

72 h). After the incubation, 3-4 colonies from each plate corresponding to different MW treatments were picked randomly, and each colony (a separate code was given to each picked colony) was streaked on to a separate nutrient agar plate. Daughter populations thus generated from a single parent colony were then inoculated into the TY broth described above for EPS quantification, and simultaneously streaked on to new nutrient agar plates.

Initially EPS estimation was made for all the MW treated inoculums. Then randomly selected colonies, from the plate corresponding to the MW treatment yielding highest EPS production, were selected for further experiments. Among these randomly picked 3-4 colonies, the highest (compared to control) EPS producer was identified as the best overproducer. Those producing EPS equivalent to control were discarded. EPS production in thus selected overproducer was studied over 3 or 4 generations until the trait of EPS overproduction was maintained by the mutant.

Statistical Analysis: All the experiments were performed in triplicate, and measurements are reported as mean ± standard deviation (SD). Statistical significance of the data was evaluated by applying *t*-test using Microsoft Excel®. *P* values less than 0.05 were considered to be statistically significant.

Results and Discussion

Results of experiments performed at 90 W: Estimation of growth and EPS production was made in three different inoculums, which received MW (90 W) treatment for 2, 4, or 6 min. Comparison of their growth and EPS production with that of control (Table 1) was made. A significant increase in EPS production was observed from the *X. campestris* culture corresponding to 6 min MW treatment; however this was not accompanied by any increase in growth. Thus, growth and xanthan production in this case seemed to be affected by MW independent of each other.

After 90 W MW treatment, the culture exposed to MW for 6 min was found to produce xanthan appreciably higher than the parent control, so it was selected for further experiments. Three colonies from the plate on to which the culture exposed to 6 min MW treatment was plated, were picked randomly and designated as 6A, 6B, and 6C. These three colonies were streaked on three separate nutrient agar plates, and the resulting population was studied for xanthan production after inoculation into TY broth (Table 2). Out of these three colonies, 6A was found to produce 43.75% higher xanthan as compared to the parent strain, accompanied by a significant increase in growth too. However, the magnitude of xanthan overproduction was much higher than the increase in growth, suggesting that xanthan overproduction was entirely not due to increased cell density in the 6A culture. The magnitude of increase in xanthan production shown by 6A was almost identical to that achieved in the first experiment for 6 min treatment (Table 1).

Further generations of 6A were then also studied for xanthan production, to investigate whether the trait of xanthan overproduction is maintained stably over generations. Table 3 lists measurements of growth and xanthan production in all the generations studied of 6A, and their comparison with those of the parent strain. The first results obtained with 6A are designated as those of generation 'zero'. MW treatment at 90 W for 6 min duration was able to alter the xanthan production in *X. campestris* to a notable extent, while growth was affected to a lesser extent. The trait of xanthan overproduction in the mutant 6A was stable only till one generation, thereafter it was able to produce xanthan equivalent to the parent strain. The mutant strain reverted back to the parent phenotype from second generation onwards.

Results of experiments performed at 450 W: Effect of MW on *X. campestris* was also investigated at 450 W, where duration of MW exposure was kept 3 min (Table 4). This MW treatment was able to induce xanthan production

in *X. campestris*, but growth was lesser than control. Four colonies from the plate on to which the culture exposed to 3 min MW (450 W) treatment was plated, were picked randomly and designated as 3A, 3B, 3C, and 3D. These four colonies were streaked on four separate nutrient agar plates, and the resulting population was studied for xanthan production after inoculation into TY broth (Table 5). Out of these four colonies, 3C was found to produce 50% higher xanthan as compared to the parent strain, although its growth experienced a decrease. This suggests that increase in xanthan production was not linked to the growth of the test organism. The magnitude of increase in xanthan production shown by 3C (Table 5) was much higher than that achieved in the first experiment for 3 min MW (450 W) treatment (Table 4).

Further generations of 3C were then also studied for xanthan production, to investigate whether the trait of xanthan overproduction is maintained stably over generations. Table 6 lists measurements of growth and xanthan production in all the generations studied of 3C, and their comparison with those of the parent strain. The first results obtained with 3C are designated as those of generation 'zero'. The trait of xanthan overproduction in the mutant 3C was inherited only upto one more generation, however the magnitude of overproduction was lesser than generation 'zero'. From second generation onwards it was able to produce xanthan equivalent to the parent strain. The mutant strain 3C reverted back to the parent phenotype from second generation onwards.

MW treatments at two different powers (90 and 450 W) employed in this study were able to alter growth and xanthan production in some of the *X. campestris* isolates obtained from MW treated suspensions. As the MW treatment was performed by placing the inoculum vial in an ice containing beaker, and the temperature did not cross 25°C, whatever alterations were observed in growth and xanthan production seem largely to have resulted from non-thermal effects of MW radiation. As the trait of xanthan overproduction was transferred from one generation of MW treated organism to the next, the MW effect can be said to be mutagenic. However this MW induced mutation was not found to be stable over multiple generations. Both the xanthan overproducing mutants (6A and 3C) obtained by two different MW treatments reverted back to the parent phenotype, suggesting the reversible nature of the MW induced mutations.

Reports suggesting reversible nature of MW effects, as well as those suggesting the MW-induced mutations to be stable, both have accumulated in literature. Disappearance of low-level MW (35 GHz, the surface of irradiated object - 30 mcW/cm^2, exposure time 10 s) induced effects after few generations in *Drosophilla melanogaster* was demonstrated by Pasiuga et al. (20). It is possible that MW treatment has a profound effect on mutation repair system of a cell for initial few generations, but later the repair system restores its efficiency. Our results, and those reported by Pasiuga et al. (20) are in contrast with few other reports describing stable mutations generated by MW treatment. For example, Lin et al. (4) obtained lactic acid overproducing mutants of *Lactobacillus rhamnosus* by using MW radiation (2450 MHz; 400 W for 3 min), and they found the mutant to be stable for increased L-lactic acid production for at least nine generations. Li et al. (13) reported enhanced cellulase production in *Trichoderma viride* mutated by compound mutagenesis using MW (2450 MHz; 700 W for 15-195 s) and ultraviolet, and these mutants showing higher cellulase production were stable up to 9 generations. Li et al (21) employed MW (250 W for 36 s) mutagenesis for obtaining *Kleibsella pneumoniae* mutants with better N_2 fixing and P-solubilizing ability, and claimed them to be genetically stable. Jangid et al. (12) found microwave treatment (2450 MHz, 800 W cm^{-2} for 1, 3, 5 and 7 s) to be capable of inducing mutations and altering gene expression in *Vigna aconitifolia*.

Increased amount of EPS production observed in the mutants obtained in the present

Table 1. Effect of different durations of MW (90 W) treatment on growth and xanthan production in *X. campestris*

Duration of MW treatment (min)	Growth(OD_{625}) (Mean ± SD)	% change compared to control	Amount of xanthan produced(g/L)	% change compared to control
0 (control)	1.28 ± 0.01	0.00	0.72 ± 0.28	0.00
2	1.29 ± 0.02	1.17	0.80 ± 0.28	11.11
4	1.28 ± 0.01	0.00	0.88 ± 0.80	22.22
6	1.26 ± 0.00	-1.56	1.04 ± 0.40	44.44*

*p<0.05; minus sign indicates a decrease over control

Table 2. Growth and xanthan gum production by three different isolates selected randomly from plate corresponding to 6 min MW (90 W) treatment

Duration of MW treatment (min)		Growth (OD_{625}) (Mean ± SD)	% change compared to control (g/L)(Mean ± SD)	Amount of xanthan produced	% change compared to control
0 (parent control)		0.89 ± 0.00	0.00	0.64 ± 0.00	0.00
6	6A	1.04 ± 0.00	16.81**	0.92 ± 0.02	43.75**
	6B	0.96 ± 0.20	7.84	0.60 ± 0.02	-6.25
	6C	1.04 ± 0.04	16.59**	0.68 ± 0.08	6.25

**p<0.01; minus sign indicates a decrease over control

Table 3. Comparison of xanthan production of the mutant 6A with parent strain over multiple generations

Generation number	Growth			Amount of xanthan produced		
	OD_{625} (Mean ± SD)		% change compared to control	(g/L) (Mean ± SD)		% change compared to Control
	Control (Parent strain)	6A		Control (Parent strain)	6A	
0	0.89 ± 0.00	1.04 ± 0.00	16.81*	0.64 ± 0.00	0.92 ± 0.02	43.75*
1	0.87 ± 0.00	0.89 ± 0.00	2.06	1.05 ± 0.00	1.47 ± 0.00	40.47*
2	1.37 ± 0.00	1.41 ± 0.01	2.91*	0.80 ± 0.00	0.85 ± 0.00	6.25
3	1.03 ± 0.01	1.05 ± 0.01	1.74	1.10 ± 0.00	1.10 ± 0.00	0.00
4	1.03 ± 0.00	1.06 ± 0.01	3.10	1.65 ± 0.00	1.70 ± 0.00	3.03

*p<0.05

Table 4. Effect of MW (450 W) on growth and xanthan production in *X. campestris*

Duration of MW treatment(min)	Growth(OD_{625}) (Mean ± SD)	% change compared to control	Amount of xanthan produced(g/L) (Mean ± SD)	% change compared to control
0 (control)	1.13 ± 0.01	0.00	1.06 ± 0.02	0.00
3	0.94 ± 0.02	-16.37*	1.30 ± 0.05	22.06*

*$p<0.05$

Table 5. Growth and xanthan gum production by four different isolates selected randomly from plate corresponding to 3 min MW (450 W) treatment

Duration of MW treatment(min)		Growth(OD_{625}) (Mean ± SD)	% change compared to control	Amount of xanthan produced(g/L) (Mean ± SD)	% change compared to control
0(parent control)		1.40 ± 0.00	0.00	0.90 ± 0.00	0.00
3	3A	1.32 ± 0.08	-5.71	0.97 ± 0.03	-7.77
	3B	1.32 ± 0.04	-5.71	1.15 ± 0.07	27.77**
	3C	1.07 ± 0.00	-23.21**	1.35 ± 0.07	50.00**
	3D	1.39 ± 0.01	-0.71	0.85 ± 0.10	-5.55

**$p<0.01$; minus sign indicates a decrease over control

Table 6. Comparison of xanthan production in the mutant 3C with parent strain over multiple generations

Generation number	Growth			Amount of xanthan produced		
	OD_{625} (Mean ± SD)		% change compared to control	(g/L) (Mean ± SD)		% change compared to Control
	Control (Parent strain)	3C		Control (Parent strain)	3C	
0	1.40 ± 0.00	1.07 ± 0.00	-23.21**	0.90 ± 0.00	1.35 ± 0.07	50.00**
1	0.75 ± 0.02	0.83 ± 0.01	9.93	1.20 ± 0.00	1.45 ± 0.02	20.83**
2	1.14 ± 0.00	1.12 ± 0.02	-1.31	2.50 ± 0.05	2.20 ± 0.50	-12.00
3	1.35 ± 0.02	1.20 ± 0.00	11.43**	1.70 ± 0.03	1.70 ± 0.05	0.00

**$p<0.01$; minus sign indicates decrease over control

study may either be due to effect of MW on xanthan synthesizing machinery of the cell, or increased secretion of xanthan through cell membrane whose permeability might have been altered following MW treatment. Exopolysaccharides are usually synthesized at the cell membrane, and then exported from the cell (22). Alteration in the permeability of cell membrane following MW treatment has been reported by Bollet et al. (23). Influx of extracellular calcium was shown to be affected by MW-induced alterations in the bacterial cell permeability (24). Calcium is an important cell signalling molecule, and calcium chloride (which we used at 0.7 g/L as one of the medium ingredients) has been one of the major quantitative factors which can affect xanthan production (25). Alteration in cell membrane permeability may contribute, in part, to the non-thermal effects of MW on microbial cells.

Growth of *X. campestris*, and EPS production by it were affected by MW differently (Table 1-6). Increase in xanthan production could not be correlated with increase or decrease in growth. This indicates that the enhancement of EPS production by the mutant strains (6A and 3C) was independent of the effect of MW on *X. campestris* growth. Enhancement in EPS production in these mutants is likely to be due to the effect of MW radiation on production and/or secretion of the EPS. Though some work has been done on xanthan biosynthesis, the export and polymerization process for it is not well understood. The xanthan synthesis is encoded by the *gum* genes located in a single gene cluster of 12 kb (2). The *gum* gene cluster codes for transferase activities in *X. campestris*, three polymerases, and also includes a gene (*gum*J) which appears to control the export of the polysaccharide. Mutation in *gum*J is lethal but blocking xanthan synthesis suppresses the lethality. Mutants deficient in *gum*J cannot export xanthan, which accumulates and kills the cells. Mutation studies have revealed a number of strains that produce xanthan with alterations in the pattern of acetylation and pyruvylation, as well as mutants which show increases in yield, rates of production and composition of the repeat sub-unit (22).

Conclusion

This study has demonstrated the ability of low power MW radiation to influence EPS production in *X. campestris*. Though the enhanced EPS production induced by MW treatment was carried over to the daughter populations of the mutants, these mutants ultimately turned back to the parent phenotype. MW energy is believed to be able to penetrate deep into the biological materials (26). The MW frequency used in this study (2450 MHz) has been shown to cause single- and double- strand DNA breaks in rat brain cells (27). Ability of MW radiation to cause DNA damage including point mutations has also been reported (4). Effective use of MW radiation for mutation experiments has been reported by many investigators (4, 5, 13, 20, 21, 28). Identifying mutagenic frequencies (with simultaneous optimization of MW power and exposure time) can pave way for large scale screening programmes employing MW mutagenesis as a tool for strain improvement, and yield genetically stable overproducing mutants. Such experiments can also provide for better understanding of the MW specific non-thermal effects.

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References

1. Morris, G. and Harding, S. (2009). Polysaccharides: Microbial. Applied Microbiology: Industrial, 482-494.

2. Vorhölter, F.J., Schneiker, S., Goesmann, A., Krause, L., Bekel, T., Kaiser, O., ... and Pühler, A. (2008). The genome of *Xanthomonas campestris* pv. campestris B100 and its use for the reconstruction of metabolic pathways involved in xanthan biosynthesis. Journal of Biotechnology, 134(1): 33-45.

3. Adrio, J.L., and Demain, A.L. (2006). Genetic improvement of processes yielding microbial products. FEMS Microbiology Reviews, 30(2): 187-214.

4. Lin, H., Chen, X., Yu, L., Xu, W., Wang, P., Zhang, X., ... and Ren, N. (2012). Screening of *Lactobacillus rhamnosus* strains mutated by microwave irradiation for increased lactic acid production. African Journal of Microbiology Research, 6(31): 6055-6065.

5. Zhang, J. Huang, L.P. Pei, Y.H. and Tian, L. (2005). Effects of mutation induced by microwave irradiation on antifungal activity of marine strain B1. Chinese Journal of Marine Drugs, 24: 1-5. (In Chinese).

6. Trivedi, N. Patadia, M., and Kothari, V. (2011). Biological applications of microwaves. International Journal of Life Sciences and Technology, 4(6): 37-46.

7. Bhattacharjee, M.K., Sugawara, K., and Ayandeji, O.T. (2009). Microwave sterilization of growth medium alleviates inhibition of *Aggregatibacter actinomycetemcomitans* by Maillard reaction products. Journal of Microbiological Methods, 78(2): 227-230.

8. Kothari, V., Patadia, M., and Trivedi, N. (2011). Microwave sterilized media supports better microbial growth than autoclaved media. Research in Biotechnology, 2(5): 63-75.

9. Sasaki, K., Honda, W., Shimizu, K., Iizima, K., Ehara, T., Okuzawa, K., and Miyake, Y. (1996). Microwave continuous sterilization of injection ampoules. *PDA* Journal of Pharmaceutical Science and Technology, 50(3): 172.

10. Mandal, V., Mohan, Y., and Hemalatha, S. (2007). Microwave assisted extraction—an innovative and promising extraction tool for medicinal plant research. Pharmacognosy Reviews, 1(1): 7-18.

11. Kothari, V., Gupta, A., and Naraniwal, M. (2012). Comparative study of various methods for extraction of antioxidant and antibacterial compounds from plant seeds. Journal of Natural Remedies, 12(2): 162-173.

12. Jangid, R.K., Sharma, R., Sudarsan, Y., Eapen, S., Singh, G., and Purohit, A.K. (2010). Microwave treatment induced mutations and altered gene expression in *Vigna aconitifolia*. Biologia Plantarum, 54(4): 703-706.

13. Li, X.H., Yang, H.J., Roy, B., Park, E.Y., Jiang, L.J., Wang, D., and Miao, Y.G. (2010). Enhanced cellulase production of the *Trichoderma viride* mutated by microwave and ultraviolet. Microbiological Research, 165(3): 190-198.

14. Rebrova, T.B. (1992). Effect of millimeter-range electromagnetic radiation on the vital activity of microorganisms. Millimetrovie Volni v Biologii i Meditcine, 1: 37-47.

15. Fregel, R., Rodriguez, V., and Cabrera, V.M. (2008). Microwave improved *Escherichia coli* transformation. Letters in Applied Microbiology, 46(4): 498-499.

16. Shamis, Y., Taube, A., Mitik-Dineva, N., Croft, R., Crawford, R.J., and Ivanova, E.P. (2011). Specific electromagnetic effects of microwave radiation on *Escherichia coli*. Applied and Environmental Microbiology, 77(9): 3017-3022.

17. Mishra, T. Kushwah, P. Dholiya, and Kothari, V. (2013). Effect of low power microwave radiation on microorganisms and other life forms. Advances in Microwave and Wireless Technology, 1(1): 4-11.

18. Dholiya K., Patel D., and Kothari V. (2012). Effect of low power microwave on microbial growth, enzyme activity, and aflatoxin production. Research in Biotechnology, 3(4): 28-34.

19. Katzen, F., Ferreiro, D.U., Oddo, C.G., Ielmini, M.V., Becker, A., Pühler, A., and Ielpi, L. (1998). *Xanthomonas campestris* pv. campestris gum mutants: Effects on xanthan biosynthesis and plant virulence. Journal of Bacteriology, 180(7):1607-1617.

20. Pasiuga, V.N., Grabina, V.A., Bykov, V.N., and Shkorbatov, Y.G. (2007). Long-term effects of low-level microwave radiation on mutation frequency in *Drosophila*. In Microwave & Telecommunication Technology, 2007. CriMiCo 2007. 17th International Crimean Conference (pp. 783-784). IEEE.

21. Li, J., Zhang, S., Shi, S., and Huo, P. (2011). Mutational approach for N_2-fixing and P-solubilizing mutant strains of *Klebsiella pneumoniae* RSN19 by microwave mutagenesis. World Journal of Microbiology and Biotechnology, 27(6): 1481-1489.

22. Van Balken, J.A.M (Ed) (1997). Biotechnological innovations in chemical synthesis. Butterworth-Heinemann.

23. Bollet, C., Gevaudan, M.J., De Lamballerie, X., Zandotti, C., and De Micco, P. (1991). A simple method for the isolation of chromosomal DNA from gram positive or acid-fast bacteria. Nucleic Acids Research, 19(8): 1955.

24. Chen, W., Hang, F., Zhao, J. X., Tian, F. W., and Zhang, H. (2007). Alterations of membrane permeability in *Escherichia coli* and *Staphylococcus aureus* under microwave. Wei sheng wu xue bao= Acta Microbiologica Sinica, 47(4): 697.

25. Mabrouk, M.E., Amani, M.D., Beliah, M.M., and Sabry, S.A. (2013). Xanthan production by a novel mutant strain of *Xanthomonas campestris*: Application of statistical design for optimization of process parameters. Life Science Journal, 10(1): 1660-1667.

26. Doran, T.J., Lu, P. J., Vanier, G.S., Collins, M.J., Wu, B., and Lu, Q.L. (2008). Microwave irradiation enhances gene and oligonucleotide delivery and induces effective exon skipping in myoblasts. Gene Therapy, 16(1): 119-126.

27. Lai, H., and Singh, N.P. (1997). Acute exposure to a 60 Hz magnetic field increases DNA strand breaks in rat brain cells. Bioelectromagnetics, 18(2): 156-165.

28. Banik, S., Bandyopadhyay, S., Ganguly, S., and Dan, D. (2006). Effect of microwave irradiated *Methanosarcina barkeri* DSM-804 on biomethanation. Bioresource Technology, 97(6): 819-823.

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Cadmium affects Phosphatase activity leading to Reduced Power assay of Barley (*Hordeum vulgare* L.)

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Abstract

Heavy metal stress is an important factor which may influence plants' growth and metabolism. The present study investigates the effects of cadmium (soil treated twice/ week with 10, 20 and 30 mM) on the specific activities of phosphatases which might lead to reducing power assay in Barley (*Hordeum vulgare* L, var 2052) seedling. A significant decrease in the redox potential of shoot compared to root was observed at the similar concentration of cadmium. A similar trend on leaves were also noted. Acid and alkaline phosphatase specific activities were significantly higher in roots than in shoot at all the three concentration of cadmium i.e. 10, 20 and 30mM, compared to controls. The above mentioned changes were more pronounced at 30mM concentration of cadmium than two other concentration. These results lead us to suggest that increased cadmium concentration in soil might lead to an adverse effects on plant growth and phosphatase activities.

Keywords: Acid phosphatase, Alkaline phosphatase, Cadmium induced stress, Hordeum vulgare, Reducing power assay.

Introduction

Barley (*Hordeum vulgare L, var RD2052*) is a staple cereal grain. It is loaded with dietary fiber and proteins for a energetic and healthy metabolism. Barley's dietary fiber is high in *beta glucan*, which helps in reducing cholesterol by binding to bile acids and removing them from the body via the feces (1). The phosphorus contents in barley plays a significant role in the different cells' structure of the body. It is also a potent antioxidants which helps in strengthening our immune system in multiple ways to prevent disease. It is also one major class of phytonutrients which has been widely studied and which include quercetin, curcumin, ellagic acid, catechins, etc (2).

Cadmium is a non-essential heavy metal that does not have any metabolic use and can be harmful even at low concentration in cereal crops. It is one of the major environmental pollutant resulting from various industrial activities, sewage sludge application and commercial phosphorous fertilizers. These activities thus part of food chain. Cadmium is widely used in electroplating, pigment, plastic stabilizers and nickel-cadmium batteries. It is known as an extremely significant pollutant due to its high toxicity and large solubility in water. The toxic effects of cadmium on biological system have been reported by several researchers. The potential risk to human health is compounded by the fact it is transferred from plant products to the human diet. At high concentrations, cadmium inhibit the growth and decrease the crop productivity (3).

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The root of plants acts as the first barrier to heavy metals in soil as it accumulates them to significantly higher concentration than do the over ground organ. Thus roots becomes the main targets for the toxic effect. Cadmium is readily taken up by the root system of many plant species and its toxicity generally is considered to be 2-20 times higher than that of other heavy metals. Cadmium also disturbs plant water relationships and often impairs photosynthesis at several physiological levels, stomatal conductance, pigments content, chloroplast structure and function (4).

Cadmium is known to cause oxidative stress due to increased production of reactive oxygen species directly via the Fenton and Haber-Weiss reaction and indirectly by inhibiting antioxidant enzyme (5). H_2O_2 particularly acts as a signaling molecule in response to cadmium and other similar stresses (6). The increase in H_2O_2 accumulation changes the redox status of the cell and induces the production of antioxidant and the activation of its mechanism. Various stressful conditions including cadmium in the growth medium, adversely affect phosphate metabolism in plants (7,8). Disruptions of metabolic processes are associated with modulation in the activities of different enzymes. Among the enzymes of phosphate metabolism which get affected include activities of phosphatases, essential for the cell regulation in many physiological processes (including maintenance of soluble phosphate) required for the normal plant growth (9).

Acid phosphatase is present in germinating seed parts and also in different cellular compartments suggesting the involvement of enzymes in various metabolic and bioenergetics events (10). Acid phosphtases show a group of enzymes that usually display broad substrate specificity whereas alkaline phosphatase show low substrate specificity .Acid and alkaline phosphatase activity is known to get inhibited by various heavy metals including nickel and cadmium.

Keeping the above in view, the present study was planned to observe the possible altechange in the physiological parameters as well as behavior of acid and alkaline phosphatase in root and shoot of barley plants growing under increasing concentration of cadmium, and also to examine reducing power assay and accumulation of cadmium in root, shoot and leaves.

Materials and Methods

The seeds of *Hordeum vulgare L, var RD2052* were procured from National Seed Corporation, New Delhi. These were surface sterilized with 0 .1% sodium hypochlorite solution for 10 min and then rinsed with distilled water. The sand was thoroughly washed with water and then treated with 2% sodium hypochlorite solution. Dried surface sterilized seeds of barley were sown in earthen pots containing equal quantities (2 Kg) of washed and acid treated loamy sand soil and the potted mixture was treated with Long Ashton nutrient solution (11). Iron was provided as Fe-EDTA. Nitrogen was given in the form of as ammonium nitrate along with nutrient solution. Cadmium was provided in the form of Cadmium Chloride in 3 concentrations (10mM, 20mM and 30mM). Cadmium treatment was given twice a week followed by irrigation with distilled water. Nutrient solution was also given twice a week. Three identical sets were maintained during the whole experiment and were conducted in green house to provide controlled experimental conditions. The samples were taken from 30 days old seedlings for biochemical analysis:

Enzyme estimation

***Acid phosphatase*:** Acid phosphatase in shoots and roots were estimated following the method of Fiske and Subbaraow (12). About 1.0g fresh tissues were homogenized in 10ml of ice cold 50 mM citrate buffer (pH 5.3) in a pre chilled pestle and mortar. Filtered through muslin cloth and then centrifuged at 10,000g for 10 min. Supernatant was used as enzyme source and incubated 3ml of substrate solution at 37°C for 5

min.To this 0.5 ml enzyme extract was added and mixed well again incubated with substrate p-nitro phenyl phosphate for 15 min at 37^{0}C. Subsequently 0.5 ml sample was drawn and mixed with 9.5 ml NaOH (0.085N). The absorbance of blank and the incubated tubes was recorded at 405 nm p-Nitro phenol (4 to 20 mM) was used as standard. Enzyme specific activity is expressed as µ mol/min /mg of protein

***Alkaline phosphatase*:** Alkaline phosphatase in shoots and roots was estimated (12) at pH (10.5) and all other conditions were same as (acid phosphatase).

***Reducing Power Assay*:** Reducing power assay associated with antioxidant enzyme system property was measured by Ferreira et.al (13) method. According to this method 2.5 ml of sample extracts were mixed with 2.5 ml phosphate buffer (pH 6.6) and 2.5 ml of 1% Potassium Ferricynide.The mixture was incubated at 50^{0}C for 20 min. A portion (2.5 ml) of TCA (10%) was added to the mixture, which was then centrifuge at 3000 rpm for 10 min .The supernatant (upper layer) of the solution 2.5 ml was mixed with distilled water (2.5 ml) and $FeCl_3$(0.5 ml,0.1%). The absorbance was measured at 700 nm and compared with the standard ascorbic acid.

***Cadmium concentration*:** 1.0 g samples (root, shoot, and leaves) were digested with concentrated nitric acid using microwave digestion system (model MDS2100CEM corporation Mathew, NC, USA). Metal analyses were formed using a Perkin Elmer atomic absorption spectrophotometer (model-AAnalyst 100). For evaluation of accuracy of analytical result, the standard Indian reference material (BND201.02 supplied by National Physical Laboratory, New Delhi, India) was analyzed together with the collected sample. Cadmium was determined at 228nm wavelength (14).

***Statistical analysis*:** Data are expressed as mean±SD.Data comparisons were carried out by using one way analysis of variance followed by Tukey HSD test (15) to compare means between the different treatment groups.

Results and Discussion

***Reducing power assay*:** Data in Table1 suggests that exposure to cadmium decreased the percent inhibition of reducing power assay. Percent inhibition in roots following exposure to cadmium (10, 20 and 30 mM) was 23.6, 61.8 and 113.0%, respectively while in shoot the inhibition was more pronounced (i.e. 62.3, 86.7, 140.2, respectively). The percent inhibition of reducing power assay in leaves also decreased following exposure to different cadmium concentrations (10mM, 20Mm, 30mM) and noted to be 17.1, 21.2 and 177.7%. Increased cadmium concentration in plants significantly decreased percent inhibition suggesting oxidative stress in exposed cell compared to normal. Under condition of oxidative stress, antioxidants play a important role in neutralizing oxidative stress in cells. In response to stress induced by heavy metals including cadmium, plants increase their proline, retinol, á-tocopherol as well as ascorbic acid contents as their redox buffer. These non-enzymatic antioxidants prevent the generation of reactive oxygen species by chelation of metals or enzyme catalyzed removal of a potential oxidant (16, 17). In this study non enzymatic antioxidants such as ascorbic acid can be described as reductant. On the other hand, inactivation of oxidants by reductant can be described as redox reaction in which one reactive species is reduced at the expense of oxidation of another. Under above conditions, antioxidant power may be referred to as reducing ability. In this context, metal activates glutathione-ascorbate cycle. It is a metabolic pathway that detoxifies hydrogen peroxide which is a reactive oxygen species that is produced as a waste product in metabolism (18).

***Acid phosphatase determination*:** Data represented in Table 2 showed that specific activities of acid and alkaline phosphatase were significantly higher in roots and shoot exposed

to 10 mM, 20 mM, 30 mM cadmium compared to control ($p<.001$). Acid phosphatase specific activity in roots was 143.51 µmol P/min/mg at 10mM concentration of cadmium compared to control. While, at the other two concentrations (20mM and 30mM) the observed activities was 170.96, 242.21 µmol P/min/mg of protein, respectively compared to control (44.79 µmol P/min/mg of protein). Specific activity of acid phosphatase increased in the shoots of plants treated with cadmium compared to control. In shoots the acid phosphatase activity was 107.14, 171.88, 253.00 µmol P/min/mg of protein, respectively at 10, 20 and 30 mM of cadmium compared to control (70.87, µmol P/min/mg of protein). Similar trend was also obtained in alkaline phosphtase. Alkaline phosphatases specific activities increased in roots of plant treated with cadmium compared to control. In roots, the noted activities were 468.63, 523.60, 718.72, µmol P/min/mg of protein, respectively at 10, 20 and 30 mM of cadmium compared to control (177.25 µmol P/min/mg of protein of protein). In shoots, the increase was less pronounced than roots. The activity in this case was 33.48, 53.50, 69.93 µmol P/min/mg of protein, respectively when exposed to 10, 20 and 30mM of cadmium respectively compared to control (17.09 µmol P/min/mg of protein). Normally, salt, heavy metals, water stresses affect the physiology and biochemistry of plant cells under in vitro and vivo conditions. These stresses are known to enhance acid phosphatase activity in pea and wheat (19). It might be due to condition of stress that the growth is restricted and delivery of phosphate is impaired, resulting in the activation of the cellular phosphatase. It thus releases soluble phosphate from its insoluble compound inside or outside the cells thereby modulating free phosphate uptake mechanism. Olmos and Hellin (20) observed that acid phosphatase are known to act under salt and water stress by maintaining a certain level of inorganic phosphate which can be co-transported with H^+ along a gradient of proton motive force. It is a major producer in roots. It is the key enzyme involved in the transport and recycling of phosphorus (21) Expression of acid phosphatase is regulated by a variety of developmental and unfavorable environmental condition.

Accumulation of cadmium in different parts of the plant: A Higher concentration of heavy metals in soils, including cadmium, could damage bio-membranes and may cause uncontrolled uptake or translocation of cadmium in plants leading to an increased accumulation of cadmium in different parts of plants. Data presented in Table 3 provides data for the accumulation of cadmium in different parts of the plants. Result indicates a significant increased cadmium concentration in root and shoots than leaves compared to control.

In roots, cadmium accumulation was 101.4, 130.43, 137.08 µg/g of fresh weight n response to exposure to 10, 20 and 30 mM of cadmium, respectively. In case of shoot, cadmium accumulation was 23.9, 44.5, 53.6 µg/g of fresh weight at 10, 20 and 30 mM of cadmium, respectively. In leaves, the accumulation was 6.0, 6.77, 13.8 µg/g of fresh weight in response to 10, 20 and 30 mM cadmium, respectively. Cadmium accumulation in stem and inactivation in root cells are probably related to its binding in cell walls, compartmentalization in vacuoles and complexation with metal binding proteins and peptides, especially phytochelatin and metallothionein (22). Cadmium is a mobile element, easily absorbed by roots and transported to shoots. It is uniformly distributed in plant organs. The level decreased in the order of roots > shoots > leaves > fruits > seeds. This distribution is due to the mobilization of the protective mechanisms of plants, which inhibits the transport to further tissues and organ (23).

Conclusion

Our results lead us to conclude that the exposure of *Hordeum vulgare* to different concentration of cadmium results in an increase in acid and alkaline phosphatase activity and redox potential. Cadmium was accumulated more in the roots of *Hordeum vulgare* compared

Table 1. Effect of cadmium on reducing power assay of *Hordeum vulgare*

Chemical	% Inhibition			
	Conc.	Root	Shoot	Leaves
Cadmium as Cadmium Chloride	10 mM	23.6 ±0.47	62.3±0.004	17.1±0.08
Cadmium Chloride	20 mM	61.8 [a] ±0.14	86.7 [a] ±0.003	21.2 [a] ±0.004
Cadmium Chloride	30 mM	113.0 [a] ±0.81	140.2 [a] ±0.94	177.7 [a] ±0.61

Values are mean ± SE of 6 observations; [a]P<0.01compared to 10 mM cadmium; % inhibition was calculated as ($\frac{\alpha - \alpha_0}{\alpha}$) x 100, where α = control value and the α_o = treated sample value.

Table 2. Effect of cadmium on acid and alkaline phosphatase activities of different plant parts of *Hordeum vulgare*

Groups	Concentration	Acid Phosphatase µmol/P /min /mg of protein		Alkaline Phosphatase (µmo/P /min /mg of protein)	
		Root	Shoot	Root	Shoot
Control	0.0 mM	44.7±0.01	70.9±0.01	177.2±0.0082	17.1±0.01
Cadmium	10 mM	143.5±0.80[a]	107.1± 0.42[a]	468.6±0.41[a]	33.5 ±0.04[a]
Cadmium	20 mM	171.0 ±0.01[a]	171.9 ±0.06[a]	523.6 [a] ±0.42[a]	53.5±0.86[a]
Cadmium	30 mM	242.2±0.81[a,b]	253.5±0.01[a,b]	718.4±0.42 [a,b]	69.9±0.26[a,b]

Values are mean ± SE of 6 observations; [a]p< 0.01 compared to control; [b]p< 0.01 compared to plants exposed to10 mM cadmium concentration

Table 3. Accumulation of cadmium in different plant parts (roots, shoot and leaves) of *Hordeum vulgare*

Groups	Concentration	Cadmium concentration (µg/g fw)		
		Roots	Shoots	Leaves
Control	0.0 mM	0.03±0.00	0.01±0.00	0.03±0.00
Cadmium	10 mM	101.4± 0.08[a]	23.9±0.081[a]	6.0±0.12[a]
Cadmium	20 mM	130.4± 0.05[a]	44.5±0.98[a]	6.77±0.04[a]
Cadmium	30 mM	137.1±1.22[a,b]	53.6±0.04[a,b]	13.8±0.12[a,b]

Values are mean ± SE of 6 observations ; [a]p < 0.01 compared to control; [b]p < 0.01 compared to 10 mM cadmium exposed group

to shoot and leaves. Cadmium also significantly affected plant growth and development with an cadmium concentrations in soils. *Hordeum vulgare* can be used as a heavy metal accumulator in heavy metal affected soils. The deleterious effects of heavy metals may be alleviated in plants if provided with appropriate concentration and forms.

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References

1. Anderson, J..W, Hanna, T.J., Peng, X. and Kryscio, R.J. (2000). Whole grain foods and heart disease risk. J Am Coll Nutr Jun 19 (3 Supple), 291S-299S.

2. Bazzano, L.A., He J., Ogden, L.G., Loria, C.M. and Whelton, P.K. (2003). Dietary fiber intake and reduced risk of coronary heart disease in US men and women: the National Health and Nutrition Examination Survey I Epidemiologic Follow-up Study. Arch Intern Med. 8, 163, 1897-904.

3. Bingham, F.T., Page, A.L., Mahler, R.J. and Ganje T.J. (1976). Yield and cadmium accumulation of forage species in relation to cadmium content of sludge-amended soil. J. Environ. Qual. 5, 57-59.

4. Stiborova, M., Ditrichova, M. and Brezinova, A. (1987). Effect of heavy metal ions on growth and biochemical characteristic of photosynthesis of barley and maize seedlings. Biol Plant 29, 453-467 .

5. Romero-Puertas, M.C., Corps, F.J., Rodriguez-Serrano, M., Gomez, M., Del Río, L.A. and Sandalio, L. M. (2007). Differential expression and regulation of antioxidative enzymes by cadmium in pea plants. J Plant Physiol 164, 1346-1357 .

6. Dat, J.F., Van Breusegem F., Vandenabeele, S., Vranová, E., Van Montagu, M. and Inez, D. (2000). Dual action of active oxygen species during plant stress responses. Cell. Mol. Life Sci. 57, 779-795.

7. Ehsanpour, A.A. and Amini, F. (2003). Effect of salt and drought stress on acid phosphatase activities in alfalfa (Medic ago sativa L) explants under in vitro culture. Afr. J. Biotech. 2, 133-135.

8. Parida, A.K. and Das, B. (2004). Effects of NaCl stress on nitrogen and phosphorus metabolism in a true mangrove Bruguiera Parviflora growth under hydroponic culture. J Plant. Physiol 161, 921-928.

9. Vance, C.P., Uhde-Stone, C. and Allan D. L. (2003). Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New Philologist 157, 432–449.

10. Bozzo, G.G., Raghothama, K.G. and Plaxton W.C. (2004). Structural and kinetic properties of a novel purple acid phosphatase from phosphate starved tomato (*Lycopersicon esculentum*) cell cultures. Biochem. J. 377, 419-424.

11. Hewitt, E.J. (1966): Sand and water culture methods used in the study of plant nutrition. Second edition. Technical communication no. 22 (Revised). Comm. Wealth Agric. Bureaux, Farnham Royal, Bucks, England, 479-534

12. Fiske, C .H. and Subbaraow, Y. (1925). The Colorimetric determination of phosphorous. J. Biol. Chem., 56, 375.

13. Ferreira, I.C.F.R., Baptista, P., Vilas-Boas M. and Barros L. (2007). Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast

Portugal: Individual cap and stipe activity. Food Chem 100, 1511-151.

14. Bremner, I. (1974). Heavy metal toxicities. Quart. Rev. Biophys 7, 75-124.

15. Lowry, R. (2008). One Way ANOVA – Independent Samples. Vassar.edu. Retrieved on December 4th.

16. Flora, S.J.S., Shrivastava, R. and Mittal, M. (2013). Chemistry and pharmacological properties of some natural and synthetic antioxidants for heavy metal toxicity. Current Medicinal Chemistry, In Press

17. Flora, S.J.S. (2009). Structural, chemical and biological aspects of antioxidants for strategies against metal and metalloid exposure. Oxid. Med. Cell. Long., 2, 191-206.

18. Foyer, C.H. and Noctor, G. (2005). Oxidant and antioxidant signaling in reevaluation of the concept of oxidative stress in Physiol. Cell and Environ. 28, 1056-1071.

19 Barret-Lennard E.D., Robson, A.D. and Greenway, H. (1982). Effect of phosphorus deficiency and water deficit on phosphatase activities from wheat leaves. J Exp Bot 33, 682 – 693.

20. Olmos, E. and Hellin, E. (1997). Cytochemical localization of ATPase plasma membrane and acid phosphatase by cerium based insalt adapted cell line of Pisum sativum. J. Exp.Bot, 48, 1529-1535.

21. Yan, X., Lia O.H., Trull, M.C., Breebe, S.E. and Lynch, J.P. (2001). Induction of a major leaf acid phosphorous availability in common bean. Plant Physiol., 125, 1901-1911.

22. Gupta, S.C. and Goldsborough, P. B. (1991). Phytochelatin accumulation and cadmium tolerance in selected tomato cell lines, Plant Physiol 97, 1306-312.

23. Gutteridge, J.M. (1994). Biological origin of free radicals, and mechanisms of antioxidant protection. Chem Biol Interact 91: 133-140.

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Decomposition of Oil Palm Bio-Waste Using Microbes

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Abstract

Conversion of oil palm biowaste namely Empty Fruit Bunches (EFB) and Mesocarp Fibre (MF) into good compost by using decomposing fungus *Trichoderma viride* was carried out in the experimental fields of the Directorate of Oil Palm Research, India. Three applications of *T. viride* fungus, in the form of broth as well as powder formulation with talc powder (Hydrated magnesium silicate) as carrier material, to oil palm Empty Fruit Bunches and Mesocarp Fibre materials resulted into fast decomposition compared to normal process. The weight gain of the treated material in the initial periods of the fungus application was mainly due to absorption of water. The fungus showed its action on decomposing the material after 15 days of application resulting in the decrease of weights. Six applications of *Trichoderma viride* fungus (three each on Empty Fruit Bunches and on Mesocarp Fibre) at monthly intervals achieved good decomposition of the cellulose material. In the first phase of decomposition the treated material were broken into small pieces and dried. In the second phase, the colour of the material changed from light brown to black. The material lost their physical structures within three months as compared to 6 months in general. Re-inoculation studies carried out using the decomposed material confirmed the results. Since the fungus *Trichoderma viride* has compatibility with other bioagents like *Metarhizium anisopliae* which causes green muscardine disease to Rhinoceros beetle, application of commercial formulations to the breeding sites enhances the decomposition of the material, thus makes faster and complete decomposition apart from controlling the rhinoceros beetle and other dreaded disease causing fungi *Ganoderma* spp. As the bioagents are environmental friendly and ecologically safe, their use for decomposition is advocated.

Keywords: Oil palm, Biowaste, Empty Fruit Bunches, Mesocarp Fibre, *Trichoderma viride.*

Introduction

Oil palm, *Elaeis guineensis* Jacq., the highest vegetable oil producing palm, has been introduced to India to fulfill the objective of self-sufficiency in edible oil production. Oil palm biomass namely Empty Fruit Bunches, oil palm fronds, male inflorescence, Mesocarp Fibre, shell and oil palm stems are waste products that are produced at the rate of 14 tonnes per hectare of plantation per annum. Oil palm Empty Fruit Bunches contain lignocelluloses and cellulose (44.2%), hemi-cellulose (33.5%), and lignin (20.4%) (1). Phenol compounds are found in lignin components and the degradation of lignin products can leach down from plant foliage/litter into the ecosystem thus have a high potential for environmental pollution (2-3). The rapid increase in the size of oil palm plantation areas throughout the world and the huge production of its waste raises concern over the disposal of these biowastes and its impact on the ecosystem structure and function (4). Processing of oil palm Fresh Fruit Bunches (FFB) results into production

of 35% bunch refuse and 15% Mesocarp Fibre which require proper disposal. Use of EFB for mulching has been proved to be practical, cost effective and substituted for mineral fertilizers in oil palm cultivation (5). In general these waste materials attract rhinoceros beetles to lay their eggs and complete their lifecycle in these heaps. The adults that are emerged out from these heaps cause severe damage to the oil palm plantations causing mortality to the palms occasionally. Hence it is essential to convert them into good compost to solve the pest problem. Termites are found feeding on pruned leaves and other dried material of oil palm and act as decomposers. Thus they are useful causing fast decomposition and improving the nutritional status of the soil by bringing sub soil to the surface to form the earthen sheaths over the feeding material (6-8).

The nutrient content of EFB averages at 0.8% N, 0.1% P, and 2.5% K and 0.2% Mg on a dry weight basis. In the past, EFB was incinerated in the mill as a means to eliminate this waste as well as to provide energy for the boilers in FFB sterilization. The bunch ash produced, which is about 6.5% by weight of the EFB, contains about 30% - 40% Potash. The ash is used as potash fertilizer and has been found to improve the yield of oil palm in Malaysia (9-10). As the direct utilization of these wastes is not advisable due to the wide C: N ratio of materials, it is necessary to convert them into good quality organic manure using different practices such as aerobic and anaerobic composting, use of microbial inoculants like *Pleurotus* spp. and *Trichoderma viride* and chemicals and vermicomposting using earthworms (11-12).

Composting has been considered as one of the alternative methods to convert organic wastes into products like carbon dioxide, water, minerals and stabilized organic matter that benefit plant growth and soil amendment (13). Nutrient contribution through organic recycling of oil palm wastes accounted for 175 kg N, 73 kg P_2O_5, 129 kg K_2O, 70 kg CaO and 71 kg MgO per ha annually. Although considerable research on composting has been conducted using EFB, Mesocarp Fibre and various organic wastes (13-16), there is less information regarding EFB composting at the field scale of operation using fungus *Trichoderma viride*.

The biological control agent, *Trichoderma viride* is very effective in managing the root rot diseases like *Ganoderma* etc. as it multiplies in the soil and offer protection throughout the crop growth. *Trichoderma* produces chitinases and β-1 glucanases, which lyses the cell wall of many fungi and thus acts as good decomposer of cellulose material. They are highly efficient producers of many extra cellular enzymes which are used commercially for production of cellulases and other enzymes that degrade complex polysaccharides. These fungi are dearer to agricultural scientists as they can help control diseases that are caused by other fungi.

Since piling up of oil palm leaves as heaps and allow them to decompose naturally (which is commonly seen practice) attracts unwanted creatures like snakes as well as rhinoceros beetles causing negative impact on the day to day activities, it is necessary to decompose them at the earliest possible. Hence the present study was taken up to find out the impact of *Trichoderma viride* on the decomposition of oil palm Empty Fruit Bunches and Mesocarp Fibre to further use it as manure.

Materials and Methods

***Experimental site*:** The experiment was carried out in the Directorate of Oil Palm Research (Indian Council of Agricultural Research), Pedavegi, Andhra Pradesh, India during 2009-10.

***Raw materials used*:** The materials used for decomposition were oil palm Empty Fruit Bunches and Mesocarp Fibre obtained from the oil palm processing factory located in Pedavegi, Andhra Pradesh. The microbial organism used for composting was *Trichoderma viride* obtained from Directorate of Oilseeds Research, Rajendranagar, Hyderabad, India. The culture

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was multiplied on *Trichoderma* specific media by isolating from the mother culture to obtain the spore count of 10^3. The spore count was calculated using Hemocytometer (neubauer).

Composting methodology: The target materials were weighed individually before treating them for composting. Fixed quantity of both EFB and Mesocarp Fibre were used as test material for treating with the fungus. A total of seven treatments including one control with four replications were maintained. Four EFBs and 1kg of Mesocarp Fibre were taken as one unit. All the seven treatments including control were applied for both EFB and Mesocarp Fibre for three months period. Observations on weight loss, physical appearance including shape and colour were recorded as parameters for conclusion (Fig. 1 and 2).

Two sets of product (fungus) formulations namely broth and commercial formulations were tested. Both the broth as well as commercial formulations of *Trichoderma viride* were serially diluted to obtain a minimum of 1000 spores per ml and was sprayed to the test material i.e. EFB and MF. Both the test materials were exposed to fungus at same concentrations with varying number of applications viz. one, two and three times at monthly intervals. To keep the test material intact in the process of decomposition, the Mesocarp Fibre was kept in fine cotton bag so as to avoid the spillage while handling. The commercial powder formulation was prepared by mixing the broth with talc powder at 1:1 ratio. While applying the fungus to the test material, it was assured that at least 1000 spores were present in each ml of solution. A fixed quantity of 100 gms or ml of fungus product was applied to each bed. Random sampling method was followed for applying the fungus on the test material. All the treatment material including control were watered daily to maintain sufficient moisture content for optimum biological activity and there by proper decomposition. Observations on the weight of the test material were taken at every 15 days after application and are tabulated. The application frequency was fixed as monthly interval. The application was done on the same material every time.

***Statistical analysis of data*:** The collected data were subjected to statistical analysis using ANACOVA for Empty Fruit Bunches and ANOVA for Mesocarp Fibre. After analyzing the data using ANACOVA and ANOVA, Critical Difference and Coefficient of Variance were calculated at 10%

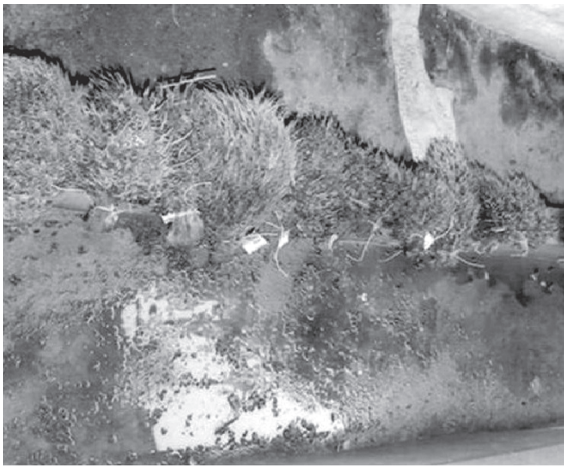

Fig. 1. Oil palm empty Fruit Bunches (pre treated)

Fig. 2. Oil palm mesocarp Fibre (pre treated)

level of significance for EFB and 5% level of significance for MF.

***Confirmation studies*:** To confirm the presence of fungus in the test material, the decomposed test materials of Empty Fruit Bunches and Mesocarp Fibre were brought to laboratory and inoculated on the *Trichoderma viride* specific media.

Results and Discussion

Observations on the physico morphological changes occurred in different treated material were compared with the pretreatment as well as untreated control material. The weights of the test material viz. Empty Fruit Bunches as well as Mesocarp Fibre from different treatments corresponding to different dates of applications were used as parameters for the decomposition. These were correlated with the pretreatment observations recorded before starting the experiment and compared with the control material. The data that was recorded on different aspects is presented in the tables 1- 7.

***Physical Characteristics of treated materials*:** Physical changes such as colour and texture of both the treated test materials viz. Empty Fruit Bunches and Mesocarp Fibre were observed during various stages of the experimental period. The final products after decomposition were grayish black in colour, with fine textured particles detached from its base material emitting earthy smell close to that of natural. The spikelets in the EFB were observed with the degraded symptoms with loosening followed by detachment from the stalk. The Mesocarp Fibre broke into pieces after discolouration from the original brown colour. At the end of the experiment, both the tested material appeared dark brown colour with pieces of irregular shape. Three applications of *T. viride* caused fragmentation of EFB into small pieces and total drying of Mesocarp Fibre. The colour of EFB and Mesocarp Fibre were in light brown before the application of fungus, but after application, the colour was turned to black (Fig. 3 and 4).

***Impact on Empty Fruit Bunches*:** Initial weights (pre treatment weights) of individual Empty Fruit Bunches (EFB) varied in different treatments indicating the variability in the material selected for experimentation. The average weights of the EFB in all the treatments including control increased in the initial period of experimentation. Regular application of water on the substrate enhanced the weights of the target material. This enhancement was observed till 30 days after initiation of the experiment in all the treated areas irrespective of treatments. Only broth treatments were observed as exception with little or no enhancement by 30 days. However in the powder treated substrates this change was not found

Fig. 3. Post treated EFB (after 3 months of experimentation) Extreme left = control

Fig. 4. Post treated Mesocarp Fibre (after 3 months of experimentation)

indicating that the action by fungus did not show much variation. The per cent change is reduced compared to control giving the indications on the action of fungus on decomposition. Where as in the observations that were recorded at 45 days after initiation clearly indicated the negative results in the weights. This was also observed in the control plots indicating that the substrates after the application of water started decomposing at 45 days period. However the per cent change in the weights of the material in different treatments indicates that the change in the weight loss compared to pretreatment was less in control (5.15) and maximum (46.31%) in the test material that received three applications of broth of fungus. The per cent decrease in the weights was in a uniform level in all the treatments till 90 days after the initiation (Table 1).

Similar trend was recorded in the treatments that received the fungus in the form of commercial powder though the per cent decrease was less to broth. In case of control plots where no fungus was applied, the weight reduction was gradual and is almost half to that of broth applied treatments indicating the efficiency of the fungus over natural

Table 1. Weight of Empty Fruit Bunches at different days of experimentation (Average of 5 replications)

Sl. No	Treatments	Pre treatment	Post Treatment					
			one application		Two applications		Three applications	
			15days	30days	45days	60days	75days	90days
1	Control	582.5	740	677.5	552.5	392	337.5	335
2	C.P.(1App)	543.75	841.25	592.5	332.5	290	251.25	248.75
3	C.P.(2Apps)	395	690	455	260	192.5	172.5	157.5
4	C.P.(3Apps)	557.5	732.5	640	447.5	341.25	166.25	136.25
5	B(1App)	550	787.5	540	337.5	280	246.25	237.5
6	B(2Apps)	410	780	546.25	235	157.5	146.25	138.75
7	B(3Apps)	507.5	697.5	393.75	272.5	188.75	140	106.25

C.P.: Commercial Powder B: Broth App.: Application

Table 2. Change (%) in EFB weights (over initial weights) at different days of experimentation (Average of 5 replications)

Sl. No.	Treatments	15DAT	30 DAT	45DAT	60DAT	75DAT	90DAT
1	Control	27.03	16.31	-5.15	-32.70	-42.06	-42.49
2	C.P. (1app)	54.71	8.97	-38.85	-46.67	-53.79	-54.25
3	C.P. (2app)	74.68	15.19	-34.18	-51.27	-56.33	-60.13
4	C.P. (3app)	31.39	14.80	-19.73	-38.79	-70.18	-75.56
5	B (1app)	43.18	-1.82	-38.64	-49.09	-55.23	-56.82
6	B (2app)	90.24	33.23	-42.68	-61.59	-64.33	-66.16
7	B (3app)	37.44	-22.41	-46.31	-62.81	-72.41	-79.06

C.P.: Commercial Powder, B: Broth, App: application DAT : Days after treatment

decomposition. At the same time three applications of fungus made the material to decompose very fast compared to single and no application (Table 2). The number of applications required for fast and complete decomposition and the difference between Commercial Powder (C.P.) as well as the Broth indicate that the three applications of the fungus i.e. *Trichoderma viride* found to cause maximum decomposition by reducing 79.06% of the weight in case of Broth and 75.56% in case of Commercial Powder.

Of the two formulations that were used, broth recorded more decomposition though not significantly different compared to commercial powder showing the same trend in all the 6 observations. The statistical analysis carried out based on ANACOVA (Table 3) indicates that all the treatment applications are significantly different from control. However two applications of C.P. and three applications of broth were found on par with each other and statistically significant at 10% level with that of other treatments. Single application of C.P. was however not significantly different to control indicating the necessity for more applications. Similar trend was found when the significance was tested for the number of applications which shows that three applications are significantly different when compared to single application (Table 4) (Fig. 2).

Impact on Mesocarp Fibre: Since fixed quantity of Mesocarp Fibre was taken for experimentation, no variation in the pre treatment weights was however observed unlike in EFB. The average weights of the Mesocarp Fibre in all the treatments including control increased till 15 days after application (Table 5) which was found varying later on. Regular application of water on the substrate enhanced the weights of the target material. This enhancement was observed till 30 days after initiation of the experiment in all the treatments except control where it was found increased even up to 45 days. Treatments with two and three applications of fungus in commercial powder formulations were found exceptions as these were showing decreased weights. In case of EFB material the change in decrease in weights was obtained with broth application. The per cent change recorded was not equal in all the treatments. It was steady in case of control where as in the commercial powder applied treatments the change was drastic as compared to broth applications. The Mesocarp Fibre is having low lignin content as compared to EFB and hence this could be the reason for quick action of fungus.

The per cent change in the weights, which is due to the decomposition action of fungus on the material, was clearly observed from 45 days after application in all the treatments. In the control, this change was observed from 60 days onwards indicating the natural phenomena of decomposition. The per cent change in the weights of the Mesocarp Fibre material in different treatments indicate that the change in the weight loss compared to

Table. 3. Analysis based on ANACOVA for treatments on EFB (10% level of significance)

Treatments	Mean weights	Grouping *
Control	553.33	A
C.P(1App)	476.46	BA
C.P.(3App)	460.42	B
Broth(1App)	448.13	B
Broth(2App)	398.13	BC
C.P(2App)	365.00	BC
Broth(3App)	333.96	C

Table. 4. Analysis based on ANACOVA for number of applications on EFB (10% level of significance)

No of applications	Mean	Grouping*
1	650.98	A
2	448.75	B
3	201.16	C
Critical Difference	54.235	

* Means with the same letter are not significantly different.

Table 5. Change (%) in Mesocarp Fibre weights (over initial weights) at different days of experimentation (Average of 5 replications)

S.No.	Treatments	15 DAT	30 DAT	45DAT	60DAT	75 DAT	90 DAT
1	Control	73.50	34.00	16.25	-3.50	-6.25	-7.50
2	C.P. (1app)	44.75	2.00	-11.50	-28.75	-30.25	-32.00
3	C.P. (2app)	51.00	-0.75	-8.75	-24.00	-26.63	-28.25
4	C.P. (3app)	28.00	-6.75	-16.25	-35.50	-41.63	-45.13
5	B (1app)	45.75	3.50	9.75	-24.75	-25.50	-26.75
6	B (2app)	59.25	12.00	-7.25	-29.25	-30.50	-32.25
7	B (3app)	57.25	6.00	-16.50	-27.75	-38.75	-43.25

DAT = Days After Treatment App = Application C.P. = Commercial Powder B = Broth

pretreatment was less in control (3.5) and maximum (35.5%) in the material that received three applications of commercial powder at 60 days after initiation of the experiment. The per cent decrease in the weights was in a uniform level in all the treatments till 90 days after the initiation of experiment. Though the commercial powder formulation has recorded quick decomposition at initial days, the broth material also made on par status at 90 days observations indicating the impact of fungus on decomposition. The per cent change in the weight reduction was 37.63 between control and three times fungus applied treatments. Similar trend was recorded in the treatments that received the fungus in the form of broth though the per cent decrease was less than commercial powder. In case of control plots where no fungus was applied, the weight reduction was gradual and was six times less than the fungus applied treatments indicating the efficiency of the fungus over natural decomposition. Three applications of fungus in the form of commercial powder made the material to decompose very fast compared to single and no application.

The observations recorded in other treatments also proved the efficacy of the fungus over control. The number of applications required for fast and complete decomposition and the difference between Commercial Powder (C.P.) as well as the Broth indicate that the three applications of the fungus i.e. *Trichoderma viride* found to cause maximum decomposition by reducing 43.25% of the weight in case of Broth and 45.13% in case of Commercial Powder. Of the two formulations that were applied on the test material i.e. Mesocarp Fibre, powder formulation recorded more decomposition though not significantly different compared to broth showing the same trend in all the 6 observations. However the per cent reduction of weights which represents the decomposition due to fungus was much less in case of Mesocarp Fibre material compared to Empty Fruit Bunches. It is not clear why the difference was occurred between these two material though all the other aspects are same. It needs to be investigated further.

The statistical analysis that was carried out based on ANOVA indicates that all the treatment applications are significantly different from that of control. However two applications of C.P. and three applications of broth were found on par with each other and statistically significant at 10% level with that of other treatments. Single application of C.P. was however not significantly different to that of control indicating the necessity for more applications. Similar trend was found when the significance was tested for the monthly

Table 6. Analysis based on ANOVA for treatments on MF (at 5% level of significance)

Treatments	Mean	Grouping*
Control	1240.00	A
Broth (two applications)	1022.08	B
Broth (single application)	1017.08	B
C.P. (two applications)	976.46	B
C.P. (single application)	958.33	B
Broth (three applications)	951.25	B
C.P. (three applications)	852.50	C
Critical Difference	87.451	

* Means with the same letter are not significantly different.

Table 7. Analysis based on ANOVA for number of applications on MF (at 5% level of significance)

Month	Mean	Grouping*
1	1292.50	A
2	1011.25	B
3	703.84	C
Critical Difference	87.451	

* Means with the same letter are not significantly different.

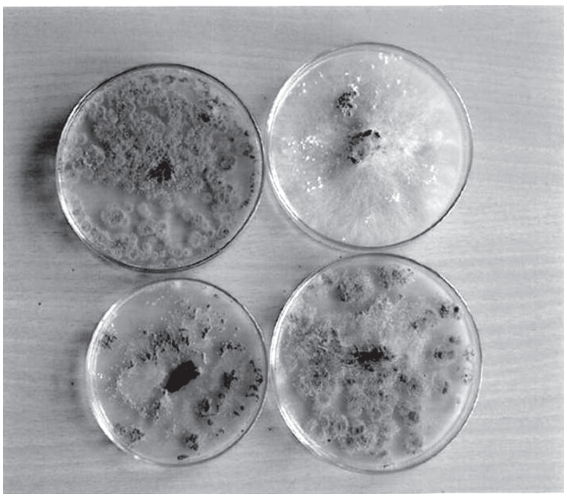

Fig. 5. Trichoderma viride reisolated from the treated EFB (top) and MF (bottom)

application which shows that three applications are significantly different when compared to single application as it reduced the weights almost 50% compared to single application. The data on statistical analysis that was carried out on the decomposition of Mesocarp Fibre are given in tables 5-7. Based on the critical difference, all the treatments found statistically significant at 5% level of significance. Three applications of powder formulation of fungus was found unique and caused significantly higher amount of decomposition compared to other treatments which were found on par with each other but superior over control. Similarly the analysis that was carried out to find the difference in monthly applications indicates that three applications were found to cause significantly (5% level) superior reduction compared to one and two applications (Table 7).

Conclusion

The fungus *Trichoderma viride* has been proved having compatibility with other bioagents like *Metarhizium anisopliae,* when applied to the breeding sites of Rhinoceros beetle which is a dreaded pest on all Arecaceae palms. The latter was observed causing green muscardine disease and there by killing the rhinoceros beetles (17) while the former was proved enhancing the decomposition of the material, thus makes faster and complete decomposition apart from controlling other dreaded disease causing fungi. The present results are in accordance with the work carried out by (18) and (19). Confirmation occurred in the reduction of weights of test material with the work carried out by latter. Hence it is concluded that *Trichoderma viride* which is a proven effective bioagent in controlling the root disease causing fungi particularly the *Ganoderma* spp. on Arecaceae palms, has once again proved as an effective and efficient fungus in decomposing oil palm waste materials such as Empty Fruit Bunches and Mesocarp Fibre. This was further confirmed by extracting the fungus from the decomposed materials of EFB and MF that was observed after three days of inoculation (Fig. 5). This confirms

that *Trichoderma viride* is a good potential fungus to use as good decomposer apart from using as good bioagent against root diseases.

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References

1. Azis A. A., Husin, M. and Mokhtar, A. (2002). Preparation of cellulose from oil palm Empty Fruit Bunches via ethanol digestion: Effect of acid and alkali catalysts. J. Oil Palm Res., 14: 9-14.
2. Kafilzadeh, F., Mohammad-Sadegh, F. and Yaghoob, T. (2010). Isolation and identification of phenol degrading bacteria from Lake Parishan and their growth kinetic assay. Afr. J. Biotechnol., 40: 6721-6726.
3. Krishna, M. V., Hrushikesh, N. Sreehari, K. Kumar, T. A. Vidyavathi, N. and Pallavi, A., (2011). Studies on bioremediation of phenol by earthworm. Int. J. Environ. Sci., 6: 1268-1273.
4. Astimar, A. A. and Wahid, M. B. (2006). Supply outlook of oil palm biomass in Malaysia. Proceedings of the Seminar on Ecomat Research and Promotion, July 24-25, Beijing, China, pp: 13-26.
5. Chee, K. H. and Chiu, S. B. (1999). Fruit waste recycling by mulching with Empty Fruit Bunches. Planter, Kuala Lumpur, 75: 435–442.
6. Kalidas, P. (1986). Studies on termites, *Odontotermes* spp. with special reference to their role in the fertility of soil. Ph.D. thesis, UAS, Bangalore, p.123.
7. Kalidas, P. and Veeresh, G. K. (1989). Plant growth in mound soils of *Odontotermes* species. Ind. J. of Agric. Sci., 59(1):8-10.
8. Kalidas, P. and Veeresh, G. K. (1990). Effect of Termite foraging on soil fertility. In *'Social Insects and the Environment'*. Oxford & IBH Publications, pp.608-609.
9. Hew, C. K. and Poon, Y. C. (1973). The effect of muriate of potash and bunch ash on yield and uptake of potassium and chlorine in oil palms on coastal soils. In Advances in Oil Palm Cultivation (R L Wastie and D A Earp, eds). Incorporated Society of Planters, Kuala Lumpur, 306-320.
10. Gurmit S., Tan, Y. P. and Ho, C. Y. (1981). Mechanical systems of bunch mulching oil palms in the Lower Perak district. In: *Proc. Natn. Workshop 'Oil palm by-product utilisation'*. Palm Oil Res. Inst. Malaysia, Kuala Lumpur, pp. 129–134.
11. Bernal, M. P., Sânchez-Monedero, M. A., Paredes, C. and Roig, A. (1998). Carbon mineralization from organic wastes at different composting stages during their incubation with soil. Agriculture, Ecosystems & Environment, 69: 175-189.
12. Kala, D. R., Rosenani, A. B. Fauziah, C. I. and Thohirah, L. A. (2009). Composting oil palm wastes and sewage sludge for use in potting media of ornamental plants. Malaysian Journal of Soil Science, 13: 77-91.
13. Baharuddin, A. S., Wakisaka, M., Shirai, Y., Abd Aziz, S., Abdul Rahman, N. A. and Hassan, M. A. (2009). Co-composting of Empty Fruit Bunches and partially treated palm oil mill effluents in pilot scale. *Int. J. Agri. Res.,* 4(2): 69-78.
14. Hock, L. S., Baharuddin, A. S., Ahmad, M. N., Shah, U. K. M., Abdul Rahman, N. A., Abd-Aziz, S., Hassan M. A. and Shirai, Y. (2009). Physicochemical changes in windrow co-composting process of oil palm Mesocarp Fibre and palm oil mill effluent anaerobic sludge. Aust. J. Basic Appl. Sci., 3(3): 2809-2816.

15. Heerden, I. V., Cronje, C., Swart, S. H. and Kotze, J. M. (2002). Microbial, chemical and physical aspects of citrus waste composting. Biores. Tech., 81(1): 71-76.

16. Khalil, A. I., Beheary, M. S., and Salem, E. M. (2001). Monitoring of microbial populations and their cellulolytic activities during the composting of municipal solid wastes," W. J. Microbiol. Biotechnol., 17(2), 155-161.

17. Kalidas, P. (2002). Integrated management practices for rhinoceros beetle affecting irrigated Oil Palm of coastal areas of India. International Oil Palm Conference (IOPC), Bali, Indonesia, July, 2002. p7.

18. Sabrina, D. T., Hanafi, M. M., Muhmud, T. M. M. and Nor azwady, A. A., (2009). Vermicomposting of Oil Palm Empty Fruit Bunch and its potential in supplying of nutrients for crop growth. Compost Science & Utilization, 17(1): 61-67.

19. Sloan Saletes, Fahri Arief Siregar, Jean-Pierre Caliman and Tony Liwang, (2004). Ligno-Cellulose Composting: Case Study on Monitoring Oil Palm Residuals. Compost Science & Utilization, 12(4): 372-382.

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Chronotherapy: Formulation and Evaluation of Pulsatile Floating Drug Delivery of Tramadol HCl

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Abstract

This study attempts to design and evaluate a chronomodulated drug delivery system of tramadol hydrochloride, it a NSAID drug for the treatment of arthritis, fibromyalgia. It was aimed to have a lag time of six hours that is, the system is taken at the bed time and expected to release the drug after a period of 6 hr at early morning time. Such time-controlled pulsatile drug delivery can be achieved mainly with cores containing tramadol hydrochloride as bioactive agent were prepared by direct compression method using different ratios of microcrystalline cellulose with effervescent agent. The tablets were coated sequentially with an inner swelling layer containing a HPMC K4M (100mg, 200mg and 300mg) and an outer rupturable layer having Eudragit RL 100 + PGE 6000 (1:1) with 1 and 2%. Which act as release controlling layers. The core serves as reservoir and the release controlling layers protect the core from the environment e.g., water, acidic pH and enzymes until the drug is released after a predetermined lag phase. Single unit rupturable pulsatile drug delivery system was chosen as the model system over erodible pulsatile drug delivery system because of ease of manufacturing, better reproducibility of the lag time and rapid drug release after a lag time. The rupture and dissolution tests were studied using the USP paddle method at 100 rpm in 0.1 N HCl for first 2hrs and then phosphate buffer pH 6.8. The lag time of the drug release decreased by increasing the inner swelling layer and increased by increasing the rupturing layer level. All the results obtained in the present study suggest that osmotic pumping effect was involved which eventually lead to the drug release.

Keywords : Pulsatile drug delivery, Rupturable layer, Eudragit RL, Tramadol Hydrochloride.

Introduction

With the advancement of the technologies in the pharmaceutical field in drug delivery systems have drawn an increasing interest over the last few decades. Nowadays, the emphasis of pharmaceutical galenic research is turned towards the development of more efficacious drug delivery systems with already existing molecule rather going for new drug discovery because of the inherent hurdles posed in drug discovery and development process.

Nowadays, concept of chronopharmaceutics has emerged, where in research is devoted to the design and evaluation of drug delivery systems that release a therapeutic agent at a rhythm that ideally matches the biological requirement of a given disease therapy (1). It is well documented that most of the body functions display circadian rhythms, e.g. heart rate, stroke volume, blood pressure, blood flow, body temperature, gastric- pH. Moreover, in a number of organs their functions vary with the time of the day (2). Diseases where a constant drug levels are not preferred, but needs a pulse of therapeutic concentration in a periodic manner acts as a push for the development of "*Pulsatile*

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Drug Delivery Systems". In these systems, there is rapid and transient release of a certain amount of drug molecules within a short time-period immediately after a predetermined off release period. Various techniques are available for the pulsatile delivery like pH dependent systems, time dependent systems, micro-flora activated systems (3), etc. which can be designed as per the physiology of disease and properties of the drug molecule. The focus of the present review is primarily on the pulsatile drug delivery methodologies and the upcoming technologies, which are being exploited on an industrial scale (4).

Pulsatile systems are gaining a lot of interest as the drug is released completely after defined lag time. Pulsatile drug delivery system is time- and site- specific drug delivery system, thus providing special and temporal delivery and increasing patient compliance (5). Pulsatile drug delivery system is defined as the rapid and transient release of certain amount of molecules within a short time period immediately after a predetermined off-release period, i.e., lag time (6)

Diseases like bronchial asthma, myocardial infarction, angina pectoris, rheumatic disease, ulcer, and hypertension display time dependence (7). Dethlefsan and Repges reported sharp increase in asthmatic attacks during early morning hours. Such a condition demands considerations of diurnal progress of the disease rather than maintaining constant plasma drug level (8). A drug delivery system administered at bed time, but releasing drug well after the time of administration (during morning hours), would be ideal in this case. Same is true for preventing heart attacks in the middle of the night and the morning stiffness typical of people suffering from arthritis.

This invention relates generally to drug delivery systems, and more particularly, to a controlled release drug delivery system which is particularly suited for use with first-pass metabolized drugs and which delivers pulsed doses at predetermined time intervals to achieve a bioavailability which is equivalent to immediate release dosage forms administered in divided doses. Morning stiffness is a very prevalent condition in most veteran persons. The trouble they experience while trying to walk, sit or do normal chores - early in the mornings inspired us to research on this project. Tramadol drug is a widely used drug in the treatment of arthritis and pain (9). The dosage of tramadol is 50mg for immediate release and can vary upto 200 mg for sustained release tablets. Since the release will be a burst release in the work, the objective is to release 50mg of NSAID – Tramadol HCl in burst release after a 6 hour lag time from time of administration after dinner. The tablet must stay in stomach for a period of 6 hours and release the drug after that time. Thus the tablet must be a floating tablet. The technique proposed to be used is rupturable delivery by coating with HPMC by direct compression and then eudragit layer by pan-coated (10).

The system proposed would be perfect because the lag time of the tablets were supposed to release on a burst release basis after a period of 6 hours. This would be possible only in rupturable chronomodulated drug delivery.

Materials and Methods

Tramadol was obtained as a gift sample from Suryakala Labs, HIG -185, KPHB. HPMC K4M, EUDRAGIT RL 100 and PEG 6000 were purchased from Sri Nihal Traders, Naryanaguda, Hyderabad. All other chemicals were of analytical grade.

Preparation of core tablets : The core tablets containing Tramadol HCl (50 mg/tablet) were prepared using the following composition show in table 1. All the excipients were mixed for 10 minutes and passed through 125 mesh size sieve and directly compressed in to 200 mg tablets (11).

Table 1. Core tablet Preparation

Constituent	Percentage (w/w)	Amount
Tramadol HCl	25%	50 mg
Citric acid	12.5%	25 mg
Micro crystalline cellulose	20%	40 mg
Starch	12.5%	25 mg
Sodium Bircarbonate	25%	50 mg
Magnesium stearate	2.5%	5 mg
Talc	2.5%	5 mg

Development of Pulsatile release tablets: Pulsatile release tablets were prepared by coating the core tablets with an inner swelling layer comprising of HPMC K4M of 100 mg, 200 mg and 300 mg respectively treated with an outer polymeric layer consisting of Eudragit RL (1% and 2%) dispersed in water/ethanol solution (60/40 v/v) using PEG 6000 (% w/w of polymer content) as a plasticizer. The Eudragit RL and PEG 6000 were taken (1:1) ratio. Inner swelling layer was applied by direct compression by recompressing the tablet on a wider die, where as outer polymeric layer was incorporated by conventional pan coating (8) with the help of customized pan coating equipment as show in table 2.

Results and Discussion

Diameter and Thickness: The diameters of the tablets were tested with the help of vernier calipers and the diameter of both core tablets and coated tablets were found to be within an error variation of 5% as show in table 3 and table 4.

Hardness: The hardness of the core tablet was tested with the help of Monsanto hardness tester and the hardness of each tablet was found to be 4.2 to 4.6 kg/cm^2 as show in table 3. The coated tablet hardness was found to be 5.4 to 5.8 kg/cm^2 as show in table 4

Friability: Friability of the tablet determines using Roche friabilator. Pre-weighed sample of tablets were placed in the friabilator and were subjected to the 100 revolutions. In present study, the friability values for all the tablet formulations were

Table 2. Development of Pulsatile release tablets

Batch Name	Direct Compression Coat	Conventional Pan Coating	Coating time
EP100-1	HPMC - 100 mg	1% (1:1) Eudragit + PEG 6000	30 min
EP100-2	HPMC – 100 mg	2% (1:1) Eudragit + PEG 6000	30 min
EP200-1	HPMC – 200 mg	1% (1:1) Eudragit + PEG 6000	30 min
EP200-2	HPMC – 200 mg	2% (1:1) Eudragit + PEG 6000	30 min
EP300-1	HPMC – 300 mg	1% (1:1) Eudragit + PEG 6000	30 min
EP300-2	HPMC – 300 mg	2% (1:1) Eudragit + PEG 6000	30 min

Table 3. Evaluation of core tablets

Batch Name	Thickness(CM)	Hardness Kg/cm^2	Frabilitity% content %	Drug time(sec)	Disintegration
EP100-1	2.8±0.02	4.2	0.0571	98±0.98	287
EP100-2	3.0±0.02	4.4	0.0637	98±0,56	306
EP200-1	3.1±0.04	4.6	0.0538	99±0.74	328
EP200-2	3.3±0.02	4.5	0.0563	99±0.89	344
EP300-1	3.5±0.03	4.6	0.0593	99±0.78	364
EP300-2	3.6±0.03	4.5	0.0408	99±0.69	376

Values as mean ± SD, n=5.

found to be <1%, indicating that the friability is within the prescribed limits (13) as show in table 3 for core tablets and table 4 for coated tablets.

Drug Content: The drug content was estimated by triturating five tablets and dissolving the approximately equivalent weight of 50 mg of tramadol HCl was extracted with 100 ml of 0.1N HCl solution and analyzed by the UV/Visible spectrophotometer at 271nm wavelength. The drug content was found to be 98 to 99 ±0.59% by using standard calibration curved (14) as show in table 3.

Disintegration time: As per the requirements of pulsatile tablets the core tablet should give rapid and transient release. The tablets prepared by using micro crystalling cellulose as diluents give disintegration time from 287 to 376 second as show in table 3.

Determination of lag time: Core tablets were coated with 100 mg, 200 mg, and 300 mg of HPMC K4M as inner swelling layer and coating with impermeable anionic polymer i.e. Eudragit and PEG 6000 with 1 and 2% the lag time of the tablet was increased. The lag time increased with increase of percentage level of coating with polymer and decreased subjected to dissolution study (15,16). The lag time was found between the ranges of 327 to 486 minutes as show in table 4.

Weight variation: The weight variation was found to be less than 0.5% before pan coating and less than 1% after coating using pan coating as show in table 5 and 6 (12).

In vitro buoyancy studies: *In vitro* buoyancy studies were performed for all the six formulations. The randomly selected tablets from each formulation were kept in a 100ml beaker containing simulated gastric fluid, pH 1.2 as per USP. The time taken for the tablet to rise to the surface and float was taken as floating lag time

Table 4. Evaluation of coated tablets

Batch Name	Thickness(CM)	HardnessKg/cm^2	Frabilitity%	Wetting time(min)
EP100-1	3.1±0.04	5.4	0.0571	327
EP100-2	3.1±0.08	5.6	0.0637	360
EP200-1	3.2±0.05	5.5	0.0538	395
EP200-2	3.3±0.07	5.6	0.0563	395
EP300-1	3.5±0.03	5.6	0.0593	424
EP300-2	3.8±0.07	5.8	0.0408	486

Values as mean ± SD, n=3.

Table 5. Weight variation test of before coating tables (mg)

S. No	EP100-1	EP100-2	EP200-1	EP200-2	EP300-1	EP300-2
1	301	302	403	399	501	502
2	302	300	401	398	502	502
3	302	301	402	402	502	500
4	302	301	399	401	502	500
5	301	302	401	400	502	501
6	301	300	402	400	500	503
Average	301.5	301	401.333	400	501.5	501.333

Table 6. Weight variation test of after coating tables (mg)

S. No	EP100-1	EP100-2	EP200-1	EP200-2	EP300-1	EP300-2
1	353	412	455	499	550	609
2	358	409	451	498	552	601
3	358	414	452	501	549	598
4	349	402	449	503	557	597
5	351	413	451	503	554	608
6	353	412	452	515	556	609
Average	353.667	410.333	451.667	503.167	553	603.667

(FLT). The duration of time the dosage form constantly remained on the surface of medium was determined as the total floating time (TFT) (table 7 and Fig.1) (17).

Table 7. *In vitro buoyancy* studies

S. No	Batch Name	Lagtime
1	EP 100-1	~ 5-6 hours
2	EP 100-2	~ after 6 hours
3	EP 200-1	~ 3-4 hours
4	EP 200-2	~3-4 hours
5	EP 300-1	~After 2 hours
6	EP 300-2	~After 2 hours

Mean ± S.D., n = 3 tablets

Fig.1. *In vitro buoyancy* studies of formulation EP 100-2 after 6hrs

Table 8. *In-vitro* drug release studies of Tramadol HCl

Time(hrs)	EP300-1	EP300-2	EP200-1	EP200-2	EP100-1	EP100-2
0.5	0	0	0	0	0	0
1	1.3	0.98	0.1	0.1	0.1	0
2	5.6	4.7	1.5	1.5	0.2	0.1
3	46.6	30.2	2.3	2.3	0.5	0.2
4	89.4	82.4	16.2	10.2	0.6	0.3
5	95.6	87.8	83.5	73.5	0.9	0.7
6	98.7	92.4	97.2	87.2	89.3	0.9
7	98.9	96.6	98.6	98.6	94.2	78.9
8	—	—	98.7	98.7	98.7	99.2
9	—	—	—	—	99.2	99.7

Mean ± S.D., n = 3 tablets

Invitro Dissolution: The *in vitro* dissolution studies were carried out by using USP XXIII dissolution type II apparatus (paddle type) at 100 rpm with temperature of 37±0.5°C. Dissolution was carried in 900 ml of 0.1N HCl for first 2 hours and continued by 6.8 pH phosphate buffer solution (18). 5 ml dissolution medium was withdrawn by pipette at periodically and replace with a fresh dissolution medium and concentration of drug tramadol HCl was determined UV visible spectrophotometer (19). The samples were analyzed at 271 nm. The drug release studies are show in table 8.

Formulation (EP300-1) and (EP300-2) achieved 3 hrs lag time with coating level 1% and 2%(1:1) Eudragit with PEG 6000 in acidic pH 1.2 followed by 89.4% and 82.4% drug release was at 4 hr of dissolution media pH 6.8 and drug release up to 7hr is 98.9% and 96.6%. From the above two formulation as concentration of coating increases the drug release is decreases. Solubility of Eudragit being well above the pH 6.8 phosphate buffer there was no considerable decrease in the drug release [20].Formulation (EP200-1) and (EP200-2) achieved 4 hrs lag time with coating level 1% and 2% (1:1) Eudragit with PEG 6000 in acidic pH1.2 followed by 83.5% and 73.5 drug release at 5 hrs of dissolution media pH6.8 phosphate buffer and drug release followed up to 8hr 98.7%. Formulation (EP100-1) achieved 6 hrs lag time with coating level 1% (1:1) Eudragit with PEG 6000 in acidic pH 1.2 followed by 89.3% drug release at 6 hrs of dissolution media pH 6.8 phosphate buffers and drug release followed up to 9hrs is 99.2%. the formulation (EP100-2) coating of 2% (1:1) Eudragit with PEG 6000, lag time was 7 hrs and drug release reduced at 78.9% and drug release found that 99.7% at 9hrs, There was burst drug release effect was observed which can be explained on the basis of the fact that as the coating concentration increased the coat became more impermeable and finally retarded the drug release (21). This may be attributed to the fact that as the coating level increased, the drug release was retarded suggesting that the thicker film formed by PEG 6000 was quiet impermeable in pH 6.8 phosphate buffer solution (22). All the results obtained in the present study suggest that osmotic pumping effect was involved which eventually lead to the drug release.

Conclusion

The objective was to achieve a drug release after a lag time of 6 hrs. The objective was successfully fulfilled with the release pattern of the drug. EP100-2 was the most efficient batch with a great lag time and burst release of the drug exactly after 6 hrs from the time of administration. Other batches especially EP200 and EP300 showed an early burst release of the drug which is not satisfactory. The tablets were successful in stopping the release for a period of time but not for 6 hrs. So, EP100 was considered the most successful batch of them all. Absolutely no amount of drug was released in during the lag time of the release. Based on the drug release pattern and the polymers used the pharmacological patterns are not altered and the drug must be able to obtain good results as the normal release. The polymers, fillers used – Eudragit RL 100, HPMC K4M, MCC, Citric acid etc are all biocompatible. So that should not cause any pharmacologic disturbances and the drug should be able to perform its normal mechanism. The inner swelling layer of HPMC K4M played an important role in lag time. It was expected that more lag time if more HPMC is coated onto the core tablet. The lowest batch EP100-1 and EP100-2 showed a release after 6 hours. And other batches EP200-1, EP200-2, EP300-1, EP300-2 showed lesser lag time. This is possibly due to extra swelling of the polymer causing the drug to be released soon. The rupture happened sooner in the EP300-2 batch which has 300mg of HPMC K4M coated onto them. By observing the lag-times the best batch was found out to be EP100-1 and EP100-2. These two batches release the drug exactly after the supposed period of 6 hours. The results of this batch were very satisfactory for being administered and pharmacological testing. This

could be the key for treatment of morning stiffness as show in fig 2.

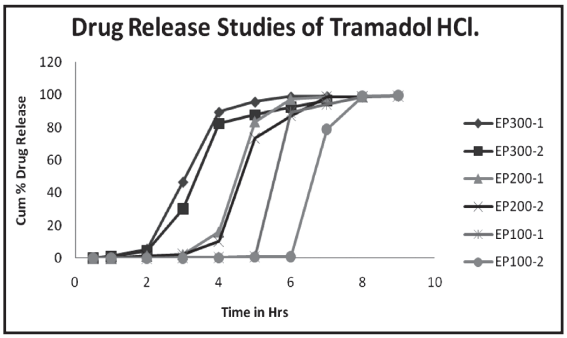

Fig.2. Drug release studies of Tramadol HCl

References

1. Harkness, J.A.L., Richter, M.B., Panayi, G.S., Pette, K.V., Unger, A., Pownall, R. and Geddawi. (1982). Circadian variation in disease activity in rheumatoid arthritis. Br Med J, 284: 551-54.

2. Dashevsky, A. and Mohamad, A., (2006). Development of pulsatile multiparticulate drug delivery system coated with aqueous dispersion Aquacoat ECD. Int. J. Pharm, 318:124-31.

3. Maroni, A., Zema, L. and Cerea, M., (2005). Oral pulsatile drug delivery systems. Expt. Opinion Drug Deli, 2: 855-71.

4. Traynor, K., Newton, D.W., Hrushesky, J.M. and Reiter, R.J. (1992). A pharmacist's primer on chronotherapeutics. American Pharmacy. NS, 32(3): 261-69.

5. Conte, U. and Maggi, L. (1996). Modulation of the dissolution profiles from Geomatrix® multi-layer matrix tablets containing drugs of different solubility. Biomaterials,17:889-96.

6. Conte, U., Maggi, L., Colombo, P and La Manna, A. (1993). Multi-layered hydrophilic matrices as constant release devices (Geomatrix® systems). J Contr Rel, 26:39-47.

7. Lamberg. L., (1991).Chronotherapeutics: Implications for drug therapy. American Pharmacy. NS 31(11): 20-23.

8. Ghebre Sellassie, I. and Knoch, A. (2002). Pelletization techniques Encyclopedia of Pharmaceutical Technology. 3rd ed. Informa Healthcare, pp.402

9. http://en.wikipedia.org/wiki/Tramadol

10. Gazzaniga, A., Busetti, C., Moro, L., Crimella, T., Sangalli, M.E., and Giordano, F. (1995). Evaluation of low viscosity HPMC as retarding coating material in the preparation of a time-based oral colon specific delivery system. Proceed Intern Symp Control Rel Bioact Mater, 22:242-43.

11. Swarbrick, J. (2005).Aqueous Polymeric Coatings for Pharmaceutical Dosage Forms. 3rd ed. Informa Healthcare. pp. 305-306.

12. USP 28-NF 23.(2005). The official Compendia of Standards, Asian Edition. United States Pharmacopoeial Convention, 626-27.

13. Indian Pharmacopoeia. Govt. of India. Ministry of Health and Family Welfare. The Indian Pharmacopoeial commission. Ghaziabad. 2007, 409.

14. Qureshi, J., Amir, M., Ahuja, A., Baboota, S. and Ali, J., (2008) Chronomodulated drug delivery system of salbutamol sulphate for the treatment of nocturnal asthma. Indian J. Pharm. Sci, 70(3): 351-56.

15. Chaudhari, H.S., Lohar, M.S., Amritkar, A.S., Jain, D.K. and Baviskar, D.T.(2011) Pulsatile drug delivery system: Review article. IJP SR, 8(2): 160 - 169.

16. Sangali, M.E., Maroni, A., Zema, L., and Gazzaniga, A. (2001) In vitro and in vivo evaluation of an oral system for time and/or site specific delivery. J Control Release, 73:103–10.

17. Sunil Kamboj. and Jagmohan oberoy. (2009). An Important Tool for Oral Controlled Release Dosage forms. Pharmainfo net, 7(6): 1 - 9

18. Youan, B. (2004). Chronopharmaceuticals: gimmick or clinically relevant approach to drug delivery. J Control Release, 98:337-53

19. Indian Pharmacopoeia, (2010). Govt. of India. Ministry of Health and Family Welfare, The Indian Pharmacopoeial commission, Ghaziabad. 2225-226.

20. Patel, G.C. and Patel, M.M. (2009). A comparative in vitro evaluation of enteropolymers for pulsatile drug delivery system. Acta Pharma. Sci, 51: 243 – 50.

21. Sahib, N.M., Abdulameer, S.A. and Rasool A.A.A. (2009). Design and in vitro evaluation of Prednisolone tablets as a potential colon delivery system. Asian J Pharm Clin Res, 2(4):84 -93

22. Bhat, A., Chowdary, K.P.R., Shobharani, R.H. and Narasu, L. (2011). Formulation And evaluation of chronopharmaceutical drug delivery of Theophylline for nocturnal asthma. Int J Pharm Pharm Sci, 3(2): 204-08.

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Evaluation of Antiarthritic Activity of *Argyreia nervosa* Leaf Extract

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Abstract

Argyreia nervosa (AN) is traditionally used in rheumatic disorders, cerebral disorders, syphilis, leucohohhrea and as wound healing agent. We aimed to validate the anti-arthritic effect of AN in Freund Complete Adjuvant (FCA) induced arthritis rats. The effects of hydroalcoholic extract of *Argyreia nervosa* leaves on the changes in rheumatoid factor (RF), Haemoglobin (Hb) content, erythrocyte sedimentation rate (ESR), prostaglandin E_2 (PGE_2), superoxide dismutase (SOD), catalase, reduced glutathione (GSH), lipid peroxidation levels and histopathology were investigated. The results showed that prophylactic treatment with *ANLE* suppressed the histopathological changes in joints and significantly reduced the FCA induced paw edema, RF, ESR, Lipid peroxidation and significantly elevated the FCA decreased SOD, Catalase and GSH levels as compared to arthritic rats. These results suggest that ANLE possess anti-arthritic activity at least in part by preventing oxidative stress.

Keywords: Antioxidant, antiarthritic activity, rheumatoid factor, Catalase, Lipid peroxidation, Superoxide dismutase, *Argyreia nervosa*.

Introduction

Rheumatoid arthritis affects about 1–1.5% of the population throughout the world, is a polyarticular inflammatory disorder that leads to joint swelling, stiffness, deformity and loss of joint function with systemic manifestations that include vasculitis, rheumatoid nodules and anaemia (1). Mediators of inflammation generate reactive oxygen species (ROS). Tumor necrosis factor – α (TNF - α), Interleukin-1β (IL-1β), interleukin-6 (IL-6), prostanoids, leukotrienes, proteases and rheumatoid factor (RF) in which cause tissue destruction of synovial membrane (2, 3). Secondarily formed radicals like hydroxylradicals destroy membrane lipids, proteins, deoxyribo nucleuc acid (DNA), hyaluronic acid and cartilage (4). Adjuvant arthritis is best experimental model not only for studying biochemistry, pharmacology of human arthritis and but also for the development of potential drugs for arthritis (5).

Argyreia nervosa (Syn: *Argyreia speciosa*) belongs to family Convolvulaceae and commonly known as Elephant creeper in English, Chandrapada, Samudrapala in Telugu, Samundar-ka pata in Hindi and Vriddhadaruka in Ayurveda (6). In Ayurvedic preparations, *AN* is used as Rasayana drug. *Argyreia nervosa* is traditionally used in Rheumatic disorders, cerebral disorders, inflammation (7), to treat wounds (8), syphilis, diarrhea (9). Leaves of *AN* are used by tribals in chittoor district of Andhra Pradesh to treat rheumatoid arthritis (10). The present study was designed to authenticate the traditional claim of *ANLE* in rheumatoid arthritis.

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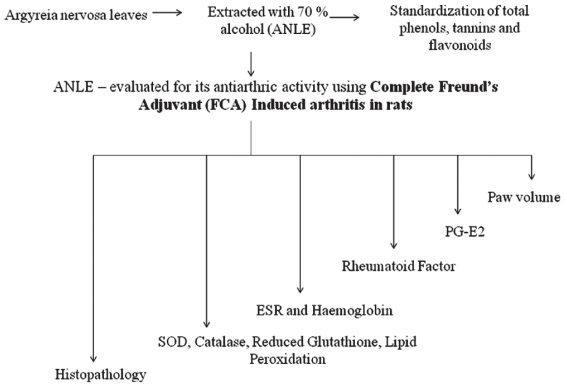


Scheme of the Proposed Hypothesis

Materials and Methods

Plant material and Hydroalcholic extraction: The leaves of *Argyreia nervosa (AN)* 1 kg were collected from Tirumala Hills in Tirupathi, Chittoor District, Andhra Pradesh, India. The plant was authenticated by Dr. Madhava Chetty, Professor, Department of Botany, Sri Venkateshwara University, Tirupati. A voucher specimen of same was already present (Voucher specimen No. 786) in the Department of Botany, S.V. University, Tirupati. The collected leaves were shade dried at room temperature and coarsely powdered, and macerated in 70% alcohol for 7 days with an intermittent vigorous shaking. Then resulted extract was dried by flash evaporation to obtain dark green residue and the percentage yield was 22.8% W/W.

Preliminary Phytochemical analysis: The total phenolic content of the extract was determined using the Folin–Ciocalteus's reagent (11). Total flavonoid content was determined using aluminium chloride ($AlCl_3$) according to the method described previously (12). IsoQuercetin was used as a standard. Total tannin content was determined by the method described earlier (13).

Animals: Pathogen free adult albino rats weighing 120-150 gm were used and housed 4 per cage in polypropylene cages under 12 hrs light/dark cycle in rooms maintained at 25±3^{0}C fed with standard laboratory chow (Hindusthan Lever Limited, Mumbai, India) and water ad libitium. Animals were acclimated to their surroundings for 10 days to eliminate the effects of stress prior to initiation of experiments. The experimental was conducted after prior approval from Institutional Ethical Committee No.1677/PO/a/12/CPCSEA.

Complete Freund's Adjuvant (FCA) Induced arthritis in rats: Adjuvant arthritis was induced by the subplantar injection of 0.1 ml Complete Freund's Adjuvant (each ml contains 1 mg of heat killed and dried *Mycobacterium tuberculosis* (strain H37Ra, ATCC 25177), 0.85 ml of paraffin oil and 0.15 ml of mannide monooleate) procured from Sigma-Aldrich, St. Louis, Mo, USA (14). The rats were randomly divided into five groups: Vehicle control (2 % gum acacia), Disease control (2 % gum acacia), ANLE (200 mg/kg/p.o), ANLE (400 mg/kg/p.o), standard Diclofenac (5 mg/kg/p.o) animals received respective treatment for 21 days and were sacrificed on day 22. The dose of ANLE was selected from the previous reports (15). The lethal dose (LD_{50}) of ANLE was more than 2000 mg/kg as none of the rats shown toxic symptoms after the dose of the extract for 14 days (15).

Paw volume assessment: Paw volumes (16) were measured with Digital Plethysmograph (Model No. 7140, UGO Basile, Comerio, Italy) on 1, 3, 6, 9, 12 and 21st day.

Biochemical measurements: On the day 22, over night fasted animals were sacrificed by diethyl ether asphyxiation and blood samples were collected by cardiac puncture. ESR was estimated by Westergren's method (17) and Haemoglobin content was estimated by the method of Sahili (18).

Prostaglandin estimation (PGE_2): Both the left control and right inflammatory hind paw were removed from above ankle and stored in Phosphate buffer containing normal saline (pH – 7) for 60 min and homogenised and centrifuged at 3000 rpm for 10 min and to 0.1 ml of

supernatant, 2ml of 0.5 mol potassium hydroxide (KOH)–methanol was added, the mixture was incubated at 50°C for 20 min, and then diluted to 20 ml with methanol. The PGE_2 level in the mixture was determined (19) with a Spectrophotometer (Model No. 177, ELICO Ltd, Hyderabad) at 278 nm and the values were expressed as µg/mg.

Rheumatoid factor (RF): Rheumatoid factor was estimated with a Commercial kit (ORGENTEC Diagnostics, Germany). To the serum sample, RF reaction buffer and RF antiserum was added according to the manufacturer's instructions. The values were expressed as IU/ml.

Superoxide dismutase: The enzyme superoxide dismutase (SOD) was determined in erythrocytes using photo-oxidation method. 3ml of packed blood cells were lysed by the addition of equal volume of cold deionised water. Hemoglobin was then precipitated by the addition of chloroform and ethanol (1.5:1). Then 500µl of water was added and centrifuged for 15 minutes at 3000rpm. The supernatant containing SOD was used for the measurement of activity. To 0.88ml of riboflavin solution ($1.3x10^{-5}$M in 0.01M potassium phosphate buffer, PH7.5), 60µl of O-dianisidine solution (10^{-2}M in ethanol) and 100ìl of clear supernatant was added and optical density was measured at 460nm. then cuvette along with reaction mixture was transferred to the illuminating box, illuminated for 4min and optical density was measured against blank containing ethanol in place of enzyme. The change in the optical density was determined (20).

Catalase: 2.5 ml of Phosphate buffer and 0.1 ml of serum was incubated at 25^0 C for 30 minutes. The absorbance was measured at 240 nm then 650µl of hydrogen peroxide solution was added to initiate the reaction. The change in absorbance was measured for 3 minutes and activity was expressed as the µmoles of H_20_2 degraded /ml/ min (21).

Reduced glutathione: To citrated blood, 5% trichloro acetic acid (TCA) solution was added and centrifuged at 3000rpm for 20 minutes. To 0.1ml of supernatant, 1ml of sodium phosphate buffer and 0.5ml of DTNB reagent were added (22). The absorbance of yellow color developed was measured at 412nm. The values were expressed in mM/ml.

Lipid Peroxidation: 0.1ml of Plasma was treated with 2 ml of 37% Thiobarbituric acid (TBA), 25N Hydrochloric acid (HCL) and 15% trichloro acetic acid (TCA) in 1:1:1 ratio and placed in water bath for 15min, cooled and centrifuged and clear supernatant was measured at 535nm against reference blank and the values were expressed in µM/ml/min (23).

Histopathological Evaluation: The hind paws were dissected out, ankles were removed and fixed in a mixture of 10% formaldehyde for 48 hrs for fixation, specimens were then placed in 5% formic acid for decalcification for 7 days. Ankles were cut into 5 µm thick sections using microtome and stained with hematoxyline and eosin (H&E) for histological evaluation using light microscopy.

Statistical analysis : All the values were expressed as Mean±SEM. The data was analyzed using Analysis of variance followed by Dunnett's multiple comparison t-test. In all the tests, the criterion for statistical significance was $P<0.05$.

Results and Discussion

By performing preliminary phytochemical analysis to ANLE, the total phenolic content was found to be 1.082 mg/g of GAE (gallic acid equivalent), flavonoid content was 0.784 mg/g of QE (isoquercetin equivalent) and the tannin content was 1.301 mg/g TAE (tannic acid equivalent).

Rheumatoid arthritis is a chronic inflammatory disease affecting about 1% of the population in developed countries. Adjuvant

induced arthritis is one of the most widely used model for studying the anti-inflammatory/anti-rheumatic properties of compounds in rats. Intra-articular injection of FCA is known to induce inflammation as well as immune response and features produced resemble rheumatoid arthritis in humans (24, 25). FCA induced arthritis is a model for chronic polyarthritis, which induces arthritis through cell-mediated autoimmunity by structural mimicry between mycobacteria and cartilage proteoglycans, which activate macrophages, lymphocytes and monokines (26). In the present study, FCA immunization resulted in swelling and erosion of joints, further bone was also destroyed. Determination of paw swelling is apparently simple, sensitive and quick procedure for the evaluation of degree of inflammation and the therapeutic efficacy of drugs. In the present study paw volume of both hind limbs were recorded on the day of FCA injection, and again measured on day 1, 3, 6, 9, 13, 21. The day 6 and 12 measurements are indicative of primary lesions and secondary lesions respectively, 1% increase in the paw volume was observed in the control paw as well indicating systemic effect of FCA immunization. On the day 21, the secondary phase of rheumatoid arthritis becomes more evident and inflammatory changes spreads systemically and becomes observable in the limb not injected with Freund's adjuvant (27, 28). Fig.1 showed the inhibitory effect of ANLE on FCA induced paw edema in rats. Rats treated with vehicle (arthritic rats) displayed visible arthritic signs on day 1 after immunization and showed maximum paw swelling on day 3, while treatment with ANLE 200 mg/kg, 400 mg/kg and diclofenac 5 mg/kg (std) significantly lowered the incidence of arthritis and markedly reduced paw swelling throughout the disease progression.

Erythrocyte sedimentation rate (ESR) is an estimation of the suspension stability of RBC's in plasma, is a nonspecific marker of disease. It is related to the number and size of the red blood cells to the concentration of plasma proteins, especially fibrinogen, α and β globulins. Acute phase proteins in ESR and C-reactive protein concentrations are elevated in response to stress and inflammation. In the present study, arthritic group of animals showed a significant increase in ESR and reduced Hb levels indicating the presence of organic disease and anaemia respectively (41). Rats treated with FCA (arthritic) showed anaemia characterized by significant decrease in the Hb levels and significant elevation in ESR (Table 1) indicating the development of arthritis, whereas treatment with ANLE 200 & 400 mg/kg and diclofenac (5 mg/kg) showed increased Hb levels and decreased ESR significantly.

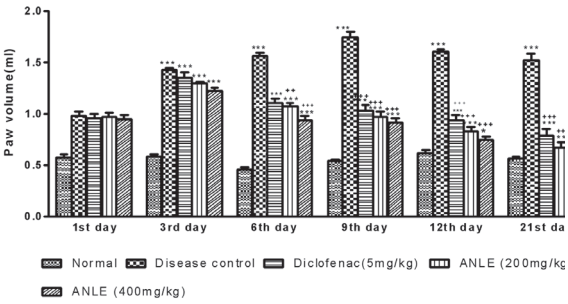


Fig. 1. Effect of ANLE on Paw edema in FCA induced Rheumatoid Arthritis

Values are expressed as Mean ±SEM [n=5]; Data were analyzed by One-way analysis of variance (ANOVA) followed by Dunnett's t-test; *($p<0.05$), **($P<0.01$), *** ($p<0.001$) Vs Disease control; +($p<0.05$), ++($P<0.01$), +++($p<0.001$) Vs Normal control.

FCA treatment increased PGE_2 levels in the arthritic rats indicating development of inflammation. PGE_2 is generated from arachidonic acid by cyclooxygenase-2 and mediates many features of inflammation (29). Proinflammatory cytokines in synovial fluid stimulate collagenase and PGE_2 production by synovial cells which inturn inhibit bone formation, stimulate resorption of proteoglycans and inhibit its biosynthesis in explants of cartilage (30). Treatment with FCA (arthritic group) showed increased PGE_2 levels significantly indicating the development of inflammation (Table 1). While the

Table 1. Effect of ANLE on Haemoglobin, Erythrocyte sedimentation rate (ESR), Prostagnandin-E_2 (PG-E_2), Rheumatoid factor (RF)

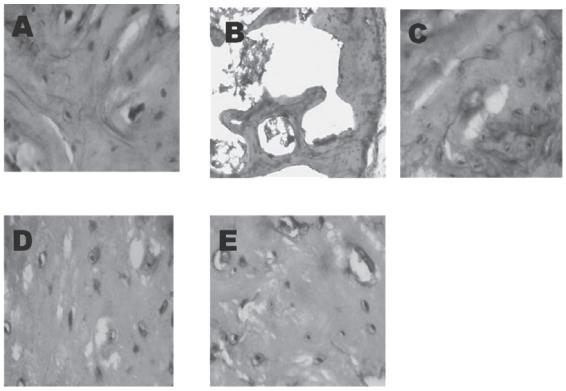
Groups	Haemoglobin(gm/dl)	ESR(mM/hr)	PG-E_2(µg/mg)	Rheumatoid Factor(RF) (IU/ml)
Normal	13.75±0.25	4.253±0.43	0.056 ±0.06	14.48 ±1.59
Disease control	9.25±0.25***	9.6±0.23***	0.174±0.06***	43.18±2.07***
Diclofenac(5mg/kg)	11.50±0.28*, +++	7.6±0.23, ++	0.123±0.50*, +++	33.73±2.37**, +++
ANLE(200mg/kg)	12.00±0.40**,+++	6.5±0.28***, +++	0.1040±0.04**, +++	26.45±2.30**, ++
ANLE(400mg/kg)	12.75±0.25 +++	5.37±0.23+++	0.082±0.03*, +++	20.33±2.15 +++

intensity of rise in PGE_2 was dose dependently reduced in ANLE 200 mg/kg & 400 mg/kg treated rats and the results were comparable with standard diclofenac (5 mg/kg) treatment group. Decrease in PGE_2 levels may be either due to inhibition of cyclooxygenase or phospholipase A_2.

RF was markedly elevated in arthritic group indicating activation of immune system and recognisation of immunoglobin G (IgG) as nonself. T-cells, IL-6, act as potent stimulus for the maturation of B cells to plasma cells to produce antibodies, serum RF recognizes immunoglobulin molecule as “non-self” results in the generation of immune complexes, which inturn bind complement and eventually lead to destruction of synovium, cartilage, and bone. Higher the levels of serum RF, higher the development of inflammation. Serum RF measures the amount of antibody IgG titre present in the serum (31, 32). ANLE dose dependently decreased PGE_2 and RF which inturn ameliorated PGE_2 and RF mediated inflammatory changes in the synovial membrane of joints. The results of histopathology confirmed it. Arthritic rats showed significant increase in the levels of Rheumatoid factor indicating FCA immunization produced arthritis. ANLE (200 and 400 mg/kg) and Diclofenac (5 mg/kg) markedly reduced the RF and values reached normal with ANLE (400 mg/kg) treatment.

The sections of the left tibiotarsal joints of normal rats (A) revealed that there was no bone matrix destruction and loss of cell integrity, Adjuvant induced arthritis group of animal (B) showed bone matrix destruction and loss of cell integrity. Diclofenac (5 mg/kg), ANLE (200 and 400 mg/kg) C, D and E respectively showed reduced bone matrix destruction and loss of cell integrity.

Fig. 2. Histological changes in hematoxylin and eosin stained tarsal joint sections in arthritis induced rats. (A) Normal (B) Disease control, (C) Diclofenac (5 mg/kg), (D) ANLE (200 mg/kg), (E) ANLE (400 mg/kg).



Values are expressed as Mean ±SEM [n=5]; Data were analyzed by One-way analysis of variance (ANOVA) followed by Dunnett’s t-test; *($p<0.05$), **($P<0.01$), *** ($p<0.001$) Vs Disease control; +($p<0.05$), ++($P<0.01$), +++($p<0.001$) Vs Normal control.

Levels of Lipid peroxidation and antioxidants SOD, GSH, Catalase were determined on day 22. SOD, GSH and Catalase levels were significantly decreased with concomitant increase in the levels of lipid

peroxidation in arthritic rats indicating development of free radicals induced oxidative stress. ANLE (200 mg/kg and 400 mg/kg) markedly elevated SOD, Catalase, GSH levels with a consistent decrease in the levels of lipid peroxidation (Table 2)

In the present study, arthritic rats showed decreased levels of SOD, GSH and Catalase indicating generation of free radicals during the development of arthritis, which was substantiated by the marked increased levels of lipid peroxides, a marker of cell damage which is online with previous results (33, 34). ROS are produced during many metabolic processes including mitochondrial respiration and enzyme activities. The superoxide anion plays a pivotal role in inflammatory joint disease. Super oxide anion is produced by one electron reduction of oxygen by several different oxidases including Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) oxidase, xanthine oxidase, cyclooxygenase as well as by several mitochondrial electron transport chain during the course of normal oxidative phosphorylation which is essential for generating adenosine triphosphate (ATP) (35). Superoxide free radical causes fibroblast proliferation and damages endothelial cells, increases the permeability of the microvasculature and promotes the migration of neutrophils to foci of inflammation and produces more aggressive free radicals (36), which is dismutated to hydrogen peroxide (H_2O_2) by manganese superoxide dismutase in the mitochondria and by copper superoxide dismutase in the cytosol (35). Superoxide also reduces iron complexes to ferrous state which reacts with hydrogen peroxide to generate hydroxyl radical by Fenton reaction. This reaction is accelerated in the synovial membrane due to high content of Iron complexes. Hydroxyl radical is extremely reactive and probably is the final mediator of most free radical induced tissue damage (37), including membrane lipids, proteins, deoxy ribo nucleic acid (DNA), cartilage, hyaluronic acid, and further damages IgG, initiates autoimmunity in rheumatoid arthritis.

Lipid peroxidation is critical mechanism of the injury that occurs in aerobic cells during RA and reflects the interaction between molecular oxygen and polyunsaturated fatty acids. This involves formation and propagation of lipid radicals, uptake of oxygen, rearrangement of double bonds, and generation of lipid peroxides cause marked alteration in the structural integrity and function of cell membranes. Lipid peroxidation by products formed under physiological and pathological conditions are scavenged by non enzymatic and enzymatic antioxidants. An imbalance between antioxidant defense mechanism and free radical generation results in cell and tissue damage (38). SOD is widely distributed in the body to protect cells against toxic effects produced by superoxide anion. Serum SOD is a valid marker of articular destruction and correlated positively with RA factor (39). Catalase is a hemo protein which catalyses the decomposition of H_2O_2 to water and oxygen, thus protecting the cell from toxic effects produced by H_2O_2 and hydroxyl radical. The main function of Catalase is to detoxify hydrogen peroxide (40). GSH is a low molecular weight thiol in the cytoplasm which is the first line of defence against peroxidation, endogenously synthesized in the liver and protects the tissue against *in vivo* toxicity of sulfhydryl – binding toxicants (40). It exists in the oxidized and reduced forms which are inter-convertible, maintains cellular level of Vitamin C in active form. Its role includes detoxication of xenobiotics, free radicals, peroxides and regulation of immune function. Low concentration of glutathione has been implicated in rheumatoid arthritis (39).

ROS modifies the rheumatoid factor immunoglobulin complex; stimulate normal neutrophils to generate further radical species. As a result hyaluronic acid depolymerises and loses its lubricant properties. The cartilage matrix is also susceptible to free radical damage, especially the polypeptide part of the proteoglycan. The enzyme catalase, released by neutrophils, plays a major function in the deterioration of rheumatoid joints. Although

Table 2. Effect of ANLE on Superoxide Dismutase (SOD), Catalase, Reduced glutathione (GSH), Lipid peroxidation.

Groups	Superoxide dismutase (IU/ml)	Catalase (µmoles/ml/ min)	Reduced (mM/ml) Glutathione	Lipid peroxidation (µM/ml/hr)
Normal	201.1 ±10.59	166.4 ±4.96	3172 ±77.38	159.3 ±23.30
Disease control	121±7.651***	60.53±3.41***	1033±102.9***	2505±100.1***
Diclofenac(5mg/kg)	163.2±3.286**, ++	79.50±2.59*, +	2447±50.5*, +++	1021±82.36*, +++
ANLE(200mg/kg)	172.9±5.81*, +++	102.1±3.49**, +++	2744±78.65**,+++	768±50.72*, +++
ANLE(400mg/kg)	180.6±5.08+++	139.9±5.65*, +++	2916±78.68+++	444.3±68.12*, +++

synovial liquid contains á-antiproteases which suppress catalase activity derived from neutrophils, the activity of these proteins can be decreased by the action of oxygen free radicals and myeloperoxidase, which was prominent in the arthritic group as observed by the decreased GSH>SOD, Catalase and increased lipid peroxides. Online with this our finding of increased lipid peroxidation and decreased antioxidants in arthritic group supports the involvement of oxidative stress in arthritis. The decreased activities of catalase, SOD, GSH may be a response to increased production of hydrogen peroxide and superoxide anion. This effect was blunte by the ANLE and diclofenac providing evidence that ANLE impart exerts its antiarthritic effect by free radical scavenging.

Phytochemical studies ANLE showed the presence of flavanoids, tannins, alkaloids, polyphenols have potent antioxidant activity (except alkaloids), which was confirmed by increased SOD, GSH, Catalase and decreased lipid peroxidation levels. As ROS is closely associated with the increase in PGE_2, RF and inflammation, free radical scavenging activity of ANLE provides close relationship between antioxidant and anti-arthritic activity. Further work is required for the clear understanding of the mechanism of action and phytochemical responsible for the activity.

Values are expressed as Mean ±SEM [n=5]; Data were analyzed by One-way analysis of variance (ANOVA) followed by Dunnett's t-test; *(p<0.05), **(P<0.01), *** (p<0.001) Vs Disease control; +(p<0.05), ++(P<0.01), +++(p<0.001) Vs Normal control.

Conclusion

The results of present study indicate that ANLE possess anti-arthritic activity in FCA model. This was demonstrated by marked decrease in paw volume, ESR, RF, PGE_2 and improvement in Haemoglobin levels. Histopathological changes also supported its antiarthritic activity. Phytoconstituents like flavanoids, phenols and tannins present in hydroalcoholic extract of ANLE may be contributing for the antiarthritic activity as they have potent antioxidant activity. Further studies can be designed on the identification of phytochemical responsible for the anti-arthritic activity.

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References

1. Mazetti, I., Griagolo, B., Roseti, L. and Dolzani, P. (2001). Differential roles of NO and oxygen radicals in chondrocytes affected by osteoarthritis and rheumatoid arthritis. Clinical Science, 101: 593–599.
2. Thabrew, M.I., Lakshmi, S., Nirma, S. and Munasinghe, C. (2001). Antioxidant potential of two polyherbal. preparations used in Ayurveda for the treatment of rheumatoid arthritis. Journal of Ethnopharmacology, 76: 285–291.

3. Brennan, F.M. and McInnes, I.B. (2008). Evidence that cytokines play a role in rheumatoid arthritis. The Journal of Clinical Investigation, 118: 3537–3545.
4. Freeman, B.A. and Crapo, J.D. (1982). Biology of disease, free radicals and tissues injury. Laboratory Investigation, 47:412-426.
5. Taurog, J.D., Argentieri, D.C. and McReynolds, R.A. (1988). Adjuvant arthritis. Methods Enzymology, 162: 339–355.
6. Gupta, A.K. and Neeraj, T. (2004). Reviews on Indian medicinal plants. Indian Council of Medical Research, New Delhi.
7. Modi, A.J., Khadabadi, S.S., Farooqui, I.A. and Deore, S.L. (2010). Argyreia speciosa Linn., Phytochemistry, Pharmacognosy and pharmacological studies. International Journal of Pharmaceutical Sciences Review and Research, 2(2): 14-21.
8. Nadkarni, K.M. (1995). Indian Materia medica. Popularprakashan private limited, Mumbai, India 1:182.
9. Gubhabakshi, D.N., Sensarma, P. and Pal, D.C. (1999). Alexicon of medicinal plant in India, New Delhi, 1: 180-181.
10. Madhava Chetty, K., Sivaji, K. and Tulasi rao, K. (2008). Flowering Plants of Chittoor District, Andhra Pradesh, India. Second edition, Tirupati, India, :219.
11. Kumar, N., Bhandari, P., Singh, B. and Bari, S.S. (2009). Antioxidant activity and ultraperformance LC-electrospray ionization-quadrupole time-of-flight mass spectrometry for phenolics-based fingerprinting of Rose species: Rosa damascene, Rosa bourboniana and Rosa brunonii. Food and Chemical Toxicology 47, 361-367.
12. Zovko Koncic, M., Kremer, D., Karlovic, K. and Kosalec, I. (2010). Evaluation of antioxidant activities and phenolic content of Berberies vulgaris L. and B. croatica Horvat. Food and Chemical Toxicology, 48: 2176-2180.
13. Bajaj, K.L. and Devsharma, A.K. (1977). A colorimetric estimation for the determination of tannins in tea. Microchimica Acta, 68: 249-253.
14. Kalia, R., Rao, C.M. and Kutty, N.G. (2007). Synthesis and evaluation of anti-inflammatory activity of N-[2-{3,5-di-tert-butyl-4-hydroxyphenyl}-4-oxo-thiazolidin-3-yl]-nicotinamide. Arzneimittel-Forschung, 57: 616-622.
15. Sunil, K.J., Chandana, V.R., Brijesh, S., Pritee, M., Sanjib, D. and Mukesh, K.D. (2011). Gastroprotective effect of standardized leaf extract from Argyreia speciosa on experimental gastric ulcers in rats. Journal of Ethanopharmacology, 137: 341-344.
16. Colpert, K.M. (1987). Evidence that adjuvant arthritis in the rat is associated with chronic pain: Pain, 28: 201-222.
17. David, G. and Sykes, A.J. (1951). Westergren and Wintrobe methods of estimating ESR compared. British Medical Journal, 2: 1496–1497.
18. Wintrobe, M.M. (1975). Clinical Hematology 7th Edition. Philadelphia: Lea and Febiger, 114-115.
19. Chen, Q. (1996). Method of Chinese Medicine Pharmacology. Chinese People Health Press, Beijing.
20. Misra, H.P. and Fridovich, J. (1979). Superoxide dismutase: a photochemical augumentation assay. Arch Biochemistry Biophysics, 181: 133-140.
21. Beers, R.F. and Sizer, I.W. (1952). A Spectrophotometric method for measuring the break down hydrogen peroxide by catalase. Journal of Biological Chemistry, 195: 133-140.
22. Ellman, G.L. (1959). Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics, 82: 70-77.
23. Nichans, W.G. and Samuelson, D. (1959). Formulation of malondialdehyde from phospholipids arachidonate during

microsomal lipid peroxidation. European journal of Biochemistry, 6: 126-130.

24. Pearson, C.M. (1956). Development of arthritis, periarthritis, periostitis in rats given adjuvants. Proceedings Society for Experimental Eiology and Medicine, 91: 95-101.
25. Sunil, S., Yuvaraj, D.M., Pallavi, R.K., Gulab, S.S., Pournima, A.S. and Amol, S.S. (2011). Antiarthritic activity of various extracts of Mesua ferrea Linn. Seed. Journal of Ethanopharmacology, 138: 700-704.
26. Van-Eden, V.W.J., Holoshitz, Z., Nevo, A., Frenkel, A. and Cohen, I.R. (1985). Arthritis induced by a T-lymphocytes clone that responds to mycobacterium tuberculosis and to cartilage proteoglycans. Proceedings of Natural Academy Sciences, 82: 5117– 5120.
27. Talwar, S., Nandakumar, K., Nayak, G.P., Punit, B., Jayesh, M.,Vijay, M., Mallikarjuna rao, C. and Richard, L. (2011). Anti-inflammatory activity of Terminalia paniculata bark extract against acute and chronic inflammation in rats. Journal of Ethnopharmacology, 134: 323-328.
28. Mithun, V.K.P., Amit, D.K. and Sucheta, D.B. (2012). Anti-arthritic and anti-inflammatory activity of *Xanthium srtumarium* L. ethanolic extract in Freund's complete adjuvant induced arthritis. Biomedicine and Aging Pathology, 2(1): 6-15
29. Serhan, C.N. and Levy, B. (2003). Success of prostaglandin E2 in structure function is a challenge for structure based therapeutics. Proceedings of National Academy of Science, 100: 8609-8611.
30. Oopman, W. (1990). Rheumatoid factor and human disease. Clinical Immunology News, 10: 137-141.
31. Mageed, R.A. (1996). The RF antigen. In van Venrooij WJ, Maini RN, editors. Manual of Biological Markers of Disease. Dordrecht7 Kluwer Academic Publishing, 1– 27.
32. Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973). Biological defense mechanism: The production by leucocytes of superoxide a potent bacteriocidal agent. Journal of Clinical Investigation, 52: 741-744.
33. Nicotera, P. and Orrenius, S. (1986). Role of thiols in protection against biological reactive intermediates. Advances in Experimental Medical Biology, 187: 41-51.
34. KizilIntuc, A., Cogalgil, S. and Cerrahoglu, L. (1998). Carnitine and antioxidant levels in patients with rheumatoid arthritis. Scand Journal of Rheumatology, 27: 441-445.
35. Evans, J.L., Goldfine, I.D., Maddux, B.A. and Grodsky, G.M. (2003). Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta – cell dysfunction? Diabetes, 52 (1): 1-8.
36. Haddad, J.J. (2002). Antioxidant and prooxidant mechanisms in the regulation of redox-sensitive transcription factors. Cell Signals, 14: 879-897.
37. Okizie, I.A. (1999). Free radicals, antioxidants International nutrition. Journal of Clinical Nutrition, 8(1): 53-63.
38. Martinez, C.M. (1995). Oxygen free radicals and human disease. Biochemistry, 77: 147-161.
39. Yuksel, A., Cengiz, M., Seven, M. and Ulutin, T. (2001). Changes in the antioxidant system and hepatic enzymes in epileptic children receiving antiepileptic drugs: two years prospective studies. Journal of Child Neurology, 16: 603-606
40. Mazzeti, I., Griagalo, B., Borzi, RM., Mcliconi, R. and Fiachini, A. (1996). Serum Cu-Zn SOD levels in patients with RA. Indian Journal of Clinical Laboratory Research, 20: 245–249.
41. Mowat, G. (1971). Haematological abnormalities in rheumatoid arthritis. Semin arthritis Rheumatology, 1: 195-199.

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Formulation and Evaluation of Oral Disintegrating Films and Tablets of Candesartan Cilexetil

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Abstract

Candesartan cilexetil is an anti-hypertensive agent. Present study is to develop Oral disintegrating films (ODFs) and Oral disintegrating tablets (ODTs) of candesartan cilexetil with an objective to resolve the swallowing problems in pediatric, geriatric patients. ODFs of candesartan cilexetil prepared by solvent casting method using film forming polymers like HPMC-E15, HPMC-5cps and HPMC 50cps. Among the prepared ODFs Q2 containing HPMC–5cps (drug: polymer ratio 1:1.5) was found to be best formulation which releases 99.98% of the drug within 15 min and disintegration time is 10 sec. ODTs prepared by direct compression method using in different concentrations of super-disintegrants . Among the prepared ODTs F3 containing crospovidone (8%) was considered to be the best formulation, which releases up to 98.23% of the drug in 30 min and disintegration time is 10.3 sec. Based on results ODFs have faster disintegration time and drug release than ODTs.

Keywords: Oral disintegrating tablets, Oral disintegrating films, Pediatrics, Super-disintegrate, Candesartan cilexetil.

Introduction

Due to increased life expectancy, the elderly constitute a major portion of the world population today (1). Due to a decline in swallowing ability with age, a great many elderly patients complain that it is difficult for them to take some currently used dosage forms such as tablets, capsules or powders (2). Oral disintegrating dosage forms are gaining prominence as new drug delivery system. These dosage forms dissolve or disintegrate in the oral cavity within a matter of seconds without the need of water or chewing. These are useful for pediatric, geriatric and also dysphasia patients leading to improved patient compliance. They are also suitable for the mentally ill, the bedridden, and patients who do not have easy access to water (3, 4). Nonetheless, oral dosing remains the preferred mode of administration for many types of medication due to its simlplicity, versatility, convenience, and patient acceptability. The techniques for preparation are commonly based on swelling, wicking of water, porosity (5-10).

Fast disintegrating films are developed by using film forming polymers (11). From the regulatory perspectives, all excipients used in the formulation of FDFs should be Generally Regarded as Safe (i.e. GRAS-listed) and should be approved for use in oral pharmaceutical dosage forms. To develop a rapidly disintegrating tablet with direct-compression method, it is necessary to find suitable excipients with good compatibility and disintegrating ability. Although the super -disintegrants primarily affect the rate of disintegration, when used at high levels, they can also affect mouth feel, tablet hardness, and friability. Thus, several factors must be considered while selecting a super disintegrant. The simultaneous presence of a disintegrant with a high swelling (or disintegrating) force, defined

as "disintegrating agent" and a substance with a low swelling force, defined as "swelling agent," was claimed as the key factor for the rapid disintegration of a tablet, also offering satisfactory physical resistance (10). Super disintegrants commonly used are crosspovidone, sodium starch glycollate and croscarmellose sodium. The mechanism of crosspovidone is mainly depending on capillary action. The mechanism of sodium starch glycollate and croscarmellose sodium is mainly depending on high swelling action (12, 13). In the present study we developed fast disintegrating tablets by using single and two different super disintegrants. We tested the effect on disintegration time, by using both capillary and high swelling mechanisms in a formulation.

Candesartan cilexetil is an antihypertensive agent. Candesartan cilexetil is an angiotensin II antagonist. In the present study we developed oral disintegrating films of candesartan cilexetil by using solvent casting method and oral disintegrating tablets of candesartan cilexetil by using direct compression technique, and tablets were tested for content uniformity, disintegration time and dissolution tests . We also compare the results of films and tablets. The films and tablets display a fast and spontaneous deaggregation in the mouth, soon after it comes in contact with saliva, dissolving the active ingredient and allowing absorption through all possible membrane it comes in contact during deglunation.

Materials and Methods

***Material*:** Candesartan cilexetil was obtained as a gift sample from Hetero Drugs, Hyderabad (India). Croscarmellose sodium, Sodium starch glycollate, Crosspovidone, Hydroxypropyl-methylcellulose E15/Cp5/Cp15 (HPMC) and Microcrystalline cellulose were gift sample from Hetero Drugs, Hyderabad (India). All chemicals and reagents used were of analytical grade.

Preparation of fast disintegrating film: Weighed quantity of HPMC E15/HPMC 50 Cps/ HPMC 5 Cps was taken in a boiling tube. To this, 16 ml of solvent mixture of dichloromethane: methanol (1:1) was added and vortexed, care should be taken to prevent the formation of lumps. The boiling tube was kept aside for 3 hours to allow the polymer to swell. After swelling, weighed quantity of candesartan cilexetil was dissolved in 4 ml of solvent mixture, added to the polymer solution and mixed well. Finally measured quantity of glycerin was added to this mixture and vortexed. It was kept aside for some time to exclude any entrapped air and was then transferred into a previously cleaned anumbra Petri plate. Drying of these films was carried out in a desiccator. The film was carefully removed from the Petri plate, checked for the imperfections and cut to the required size to deliver the equivalent dose (3.79 cm^2) per strip. Film samples with air bubbles, cuts, or imperfections were excluded from the study.

Preparation of fast disintegrating tablet: All the required ingredients were passed through 40 mesh to get uniform size particles and weighed accurately. Whole amount of drug, Pearlitol SD 200, Avicel pH 101, sodium saccharine and flavour except lubricant were mixed in the increasing order of their weights in a mortar. To this mixture Talc and Sodium stearyl fumarate were added. The final mixture was shaken manually for 5-10 minutes in a plastic bag. This powder was passed through the hopper of 16 station rotary tabletting machine and punched into tablets using 5 mm s/c. The process is similar for all the formulations, which are prepared by direct compression technique.

Evaluation of films

Thickness: The thickness of the film (3.79 cm^2 cm) was measured at four different points on one film using screw gauge. For each formulation ten selected films were used and average thickness was recorded (14).

Weight variation test: 20 films were randomly selected from each formulation and their average weight was calculated using digital balance. Individual weight of each film was also noted using the same and compared with the average weight.

Content uniformity test: The formulated polymeric films were assayed for drug content in each case. Three polymeric films from each formulation were assayed for content of drug. Films from each formulation were taken, and were allowed to dissolve in 100 ml distilled water on a rotary shaker. The solution was diluted suitably and the absorbance of the solution was measured using UV-Vis spectrophotometer at a wavelength of 205nm against distilled water as blank (14-16).

Evaluation of tablets

Pre compression parameters

***Preformulation study*:**It is the first step in rational development of dosage forms of drug substance. Pre formulation testing is defined as investigation of physical and chemical properties of a drug substance alone and when combined with excipients. It gives information needed to define the nature of the drug substance and provide frame work for the drug combination with pharmaceutical excipients in the dosage form.

***Bulk Density (D_b)*:**It is the ratio of total mass of powder to the bulk volume of powder (17). It was measured by pouring the weight powder (passed through standard sieve # 20) into a measuring cylinder and initial weight was noted. This initial volume is called the bulk volume. From this the bulk density is calculated according to the formula mentioned below. It is expressed in g/ml and is given by

$$D_b = M / V_b$$

Where,

M is the mass of powder

V_b is the bulk volume of the powder.

***Tapped Density (D_t)*:**It is the ratio of total mass of the powder to the tapped volume of the powder (17). Volume was measured by tapping the powder for 750 times and the tapped volume was noted if the difference between these two volumes is less than 2%. If it is more than 2%, tapping is continued for 1250 times and tapped volume was noted. Tapping was continued until the difference between successive volumes is less than 2 % (in a bulk density apparatus). It is expressed in g/ml and is given by

$$D_t = M / V_t$$

Where,

M is the mass of powder

V_t is the tapped volume of the powder.

***Carr's index (or) % compressibility*:** It indicates powder flow properties (17). It is expressed in percentage and is give

$$I = \frac{Dt - Db}{Dt} \times 100$$

Where,

D_t is the tapped density of the powder and

D_b is the bulk density of the powder.

***Hausner ratio*:** Hausner ratio is an indirect index of ease of powder flow (17). It is calculated by the following formula.

$$\text{Hausner ratio} = D_t / D_b$$

Where,

D_t is the tapped density.

D_b is the bulk density.

Lower hausner ratio (<1.25) indicates better flow properties than higher ones (>1.25).

***Angle of Repose*:** The friction forces in a loose powder can be measured by the angle of repose (π) (17). It is an indicative of the flow properties of the powder. It is defined as maximum angle possible between the surface of the pile of powder and the horizontal plane

$$\tan(\theta) = h / r$$

$$\theta = \tan^{-1}(h / r)$$

Where, θ is the angle of repose.

h is the height in cms

r is the radius in cms.

The powder mixture was allowed to flow through the funnel fixed to a stand at definite height. The angle of repose was then calculated by measuring the height and radius of the heap of powder formed. Care was taken to see that the powder particles slip and roll over each other through the sides of the funnel. Relationship between angle of repose and powder flow property.

Post compression parameters (18-20)

Weight variation test **:** 20 tablets were randomly selected from each formulation and their average weight was calculated using digital balance. Individual weight of each tablet was also calculated using the same and compared with the average weight. The Mean ± S.D. were noted. The tablets meet USP specifications if no more than 2 tablets outside the percentage limit and if no tablet differs by more than 2 times the percentage limit.

Thickness measurement **:** Randomly 10 tablets were taken from each formulation and their thickness was measured using a screw gauge. The individual tablet was placed between two anvils of the screw gauge and sliding knob was rotated until the tablet was tightly fitted. The reading was noted. The Mean ± S.D. were noted. The tablet thickness should be controlled within a ± 5% variation of standard value.

Hardness and Friability**:** The tablet hardness of different formulations was measured using the Monsanto hardness tester. The tester consists of a barrel containing a compressible spring held between two plungers. The lower plunger was placed in contact with the tablet, and a zero was taken. The upper plunger was then forced against the spring by turning a threaded bolt until the tablet fractures. As the spring is compressed, a pointer rides along a gauge in the barrel to indicate the force. The force of fracture is recorded, and the zero force reading is deducted from it. Generally, a minimum hardness of 4 kg is considered acceptable for uncoated tablets. The hardness for ODTs should be preferably 3 kg (18-20).

Method (Friability)**:** This test is performed using a laboratory friability tester known as Roche Friabilator. 10 tablets were weighed and placed in a plastic chambered friabilator attached to a motor, which revolves at a speed of 25 rpm, dropping the tablets from a distance of 6 inches with each revolution. The tablets were subjected to 100 revolutions for 4 minutes. After the process, these tablets were dedusted and reweighed. Percentage loss of tablet weight was calculated.

$$\% \text{ Friability} = \frac{(w1-w2)}{w1} \times 100$$

Where,

W1 = Initial weight of the 20 tablets before testing.

W2 = Final weight of the 20 tablets after testing.

Friability values below 1% are generally acceptable.

Assay**:** 20 tablets were randomly selected, weighed and finely powdered and quantity of powder equivalent to one tablet was added to 100 ml solvent mixture of pH 6.8 phosphate buffer and methanol (1:1) in a conical flask. Conical flasks were placed on a rotary shaker overnight. An aliquot of solution was centrifuged and supernatant was filtered through a 0.22µ filter. Absorbance of the resulted supernatant solution was measured using U.V-Visible spectrophotometer at a wavelength of 205nm against pH 6.8 phosphate buffer as blank. Concentrations were calculated with the help of standard graph and total amount present in the formulation was calculated.

Wetting time and Water absorption ratio (R) **:** Five circular tissue papers were placed in a Petri plate with a 10 cm diameter. Ten milliliters of water containing eosin, a water-soluble dye, was added to the Petri plate. The dye solution is used to identify the complete wetting of the tablet surface. A tablet was carefully placed on the surface of tissue paper in the Petri plate at room temperature. The time required for water to reach the upper surface of the tablets and completely wet them was noted as the wetting time. To check for reproducibility, the measurements were carried out in replicates (n=6). The wetting time was recorded using a stopwatch.

The weight of the tablet before keeping in the Petri plate was noted (W_a) using Shimadzu digital balance. The wetted tablet from the Petri

plate was taken and reweighed (W_b) using the same. The Water absorption ratio, R, was determined according to the following equation:

$$R = 100 (W_b - W_a) / W_b$$

Where W_a and W_b are the weight before and after water absorption respectively.

In vitro disintegration studies film/tablet **:** Disintegration time gives an indication about the disintegration characteristics and dissolution characteristics of the film/tablet. The film/tablet was placed in a Petri dish containing 10 ml distilled water. The time required for the film to break was noted as *in vitro* disintegration time (9, 18-20).

In vitro dissolution studies film/tablet **:** Dissolution test was carried out using USP rotating paddle method (apparatus II). The stirring rate was 50 rpm. 6.8 pH phosphate buffer and methanol (1:1) was used as dissolution medium (900 ml) and was maintained at 37 ± 1^0C. Samples of 5ml were withdrawn at predetermined intervals (2, 4, 6, 8, 10, 15, 20, 25 and 30 min), filtered and replaced with 5ml of fresh dissolution medium. The collected samples were suitably diluted with dissolution fluid, where ever necessary and were analyzed for the candesartan cilexetil at 205 nm by using UV spectrophotometer. Each dissolution study was performed for three times and mean values were taken (9, 18-20).

Results and Discussions

The objective of the present study is to design the oral disintegrating candesartan cilexetil films and tablets that disintegrate or disperse in the saliva within few seconds of its application.

Films **:** The Oral disintegrating films were prepared by solvent casting technique using HPMC – E15, HPMC – 50cps and HPMC – 5cps (Table 1). The strips were evaluated for drug content, film thickness, average weight, *in vitro* disintegration time, *in vitro* dissolution studies. Results were formulated in (Table 2). Assay was performed and percent drug content of all the batches were found to be 104.19 ± 0.71, 99.40 ± 0.74 and 101.82 ± 0.16 of candesartan cilexetil which was within the acceptable limits. All the batches were evaluated for thickness using screw gauge. As all the formulations contain different amount of polymers, hence the thickness was gradually increases with the amount of polymers. The thickness was found to be 0.47 ± 0.04 - 0.79 ± 0.05 mm.

In vitro disintegration time of films **:** Disintegration test was performed for all the batches and the disintegration time was recorded less than 30 sec for all batches. The disintegration time of formulation Q2 containing HPMC 5 Cps was found to be 10 sec and was selected as the best ODF formulation among 3 formulations

In vitro dissolution studies of films **:** In *vitro* dissolution studies of the prepared ODFs was performed in mixture of solvent pH 6.8 phosphate buffer and methanol (1:1) using USP dissolution apparatus type 2. Results showed all the batches release more than 90% of drug within 15 min. Among 3 ODF formulations Q2 was found to be best formulation because it disintegrates within 10 sec and it showed 99.98 ± 0.19 % drug release within 15 min.

Tablets **:** Using various super disintegrants like Crospovidone , Croscarmellose sodium, Sodium starch glycolate tablets were prepared along with other additives. Direct compression method was used for the preparation of tablets. A total number of 18 formulations were prepared and evaluated (Table 3, 4). To achieve rapid disintegration time, most of the excipients selected must be water soluble by nature. Pearlitol SD 200 is a directly compressible grade of mannitol with good flow properties and gives a refreshing or cooling effect in the mouth due to its negative heat of solution. This excipient was used as a bulking agent to achieve the desired tablet weight. Avicel 101 was included in the formulation mainly as a disintegrant at the concentrations used and to

Table 1. Formulations of films

Ingredients	For 1 petriplate						
	HPMC- E15			HPMC-5Cps		HPMC-50Cps	
	P1 (1:1.25)	P1 (1:1.5)	Q1 (1:1.25)	Q2 (1:1.5)	R1 (1:1.25)	R2 (1:1.5)	R3 (1:1.75)
candesartan cilexetil(mg)	74.76	74.76	74.76	74.76	74.76	74.76	74.76
Polymer (mg)	93.45	112.14	93.45	112.14	93.45	112.14	149.52
Glycerin (µl)	10	10	10	10	10	10	10
Sodium saccharine (mg)	10	10	10	10	10	10	10
Orange flavor (mg)	10	10	10	10	10	10	10
Methanol:dichloromethane(ml)	20	20	20	20	20	20	20

All batches were casted on Petri plate to provide 8 strips with dimension 3.79 cm^2 after drying.

Table 2. Physical evaluation of films

Formulation code	Thickness[a] (mm)	Average weight[a] (mg)	Disintegration time[a] (sec)	Drug content[b] (%)
P2	0.47±0.04	12.85±0.1	13.80±1.05	104.19±0.71
Q2	0.79±0.05	13.49±0.07	10.00±0.08	99.40±0.74
R3	0.52±0.06	12.9±0.08	29.71±0.95	101.82±0.16

a: Mean ± S.D., n = 6 tablets , b: Mean ± S.D., n = 5

Table 3 . Formulation codes for ODT'S

Disintegrant used	Concentration (%)	Formulation code
Crosspovidone	468	F1F2F3
Croscarmellose sodium	468	F4F5F6
Sodium starch glycolate	468	F7F8F9
Crosspovidone +Croscarmellose sodium	468	F10F11F12
Croscarmellose sodium +Sodium starch glycolate	468	F13F14F15
Sodium starch glycolate + Crosspovidone	468	F16F17F18

some extent as diluents. This grade of Microcrystalline cellulose is powder in nature and thus displays excellent flow. To impart pleasant taste and mouth feel Sodium Saccharin and orange flavor were included as sweetening and flavoring agents respectively. Sodium Stearyl Fumarate was employed as a lubricant instead of magnesium stearate to overcome the metallic taste of the latter and also due to its water soluble nature.

Crospovidone polymers are densely crosslinked homopolymers of N – vinyl 2 – pyrrolidones. Their porous particle morphology helps to rapidly wick liquids into the tablet by capillary action to generate the rapid volume

expansion and hydrostatic pressures that cause tablet disintegration. In addition to its unique particle size and morphology, Crospovidone is non ionic and its disintegration performance will neither be influenced by pH changes in the gastrointestinal tract nor will they complex with ionic drug actives. They can also be used as solubility enhancers resulting in a faster dissolution rate without forming gels.

Croscarmellose sodium is crosslinked carboxymethyl cellulose sodium which can be used at concentrations of upto 5% as a disintegrant. Its unique fibrous nature gives excellent water wicking capabilities and crosslinking makes it hydrophilic and highly absorbent material, resulting in its swelling properties. It rapidly swells upto 4 – 8 times its original volume on contact with water. Like Crospovidone, it is also used as a dissolution aid, hence the name Ac-Di-Sol (accelerates dissolution). Sodium Starch Glycolate is a sodium salt of carboxymethyl ether of starch,usually employed at concentrations between 2 – 8% although an optimum concentration of 4% may sufficient in many cases. Disintegration occurs by rapid uptake of water followed by rapid and enormous swelling, which is its primary mechanism of action. It (explotab) swells upto 300 times its original volume in water.

***Pre-formulation studies*:** The results obtained by evaluating the powder blends of drug and excipients are shown in table. Bulk density and tapped density were found in the range of 0.362 – 0.391 g/cc and 0.436 – 0.466 g/cc respectively. The value of Hausner was in between 1.17 – 1.20 (< 1.25) indicating that all batches of powder blends were having good compressibility. Values of angle of repose (è) was found in the range of 24.87 - 29 showing that blend of powder was free flowing and can be used for direct compression.

In all formulations, tablet weight and thickness were within mean ±7.5% and mean ±5% respectively. The average weight in all the eighteen formulations was found to be 79.65 ± 0.77 mg to 80.13 ± 0.87 mg. The thickness varies between 3.71 ± 0.01 to 3.85 ± 0.24 mm. Friability values were less than 1% in all cases. Hardness of all the tablets was maintained at 2.73 ± 0.08 to 3.5 ± 0.15 kg/cm^2 for all the formulations as mentioned before. Assay was performed and percent drug content of all the tablets were found to be between 89.23 ± 1.22% and 102.00 ± 0.21% of candesartan cilexetil, which was within the acceptable limits. Wetting time was determined for all the formulations. The values lie between 12.87 ± 0.22 to 59.90 ± 0.67sec. The variability in wetting time for different formulations may be due to the changes in the compaction which cannot be controlled during tablet preparation and the type of the disintegrant affected the wetting of the tablets. On comparing the superdisintegrants the formulations containing Crospovidone and Croscarmellose sodium + sodium starch glycolate take less wetting time than the other formulations. Water absorption ratio ranged from 26.38 ± 0.33 % to 172.80 ± 0.22 %. Crosspovidone and Crosscarmellose sodium perform their disintegrating action by wicking through capillary action and fibrous structure, respectively with minimum gelling. The relative ability of the various disintegrants to wick water into the tablets was studied. After contact with water the tablets containing sodium starch glycolate swelled, the outer edge appeared gel like. Tablets containing Crosspovidone quickly wicks water and were hydrated, but were soft as compared with tablets prepared with Crosscarmellose sodium and sodium starch glycolate. The center of the tablets with Sodium starch glycolate and Croscarmellose sodium remained dry and hard.

***Disintegration time of tablets* :** Disintegration time is considered to be important criteria in selecting the best ODT formulation. The *in vitro* disintegration time for all the 18 formulations varied from 10.30 ± 0.6 to 34.55 ± 0.88 seconds. The rapid disintegration was seen in the formulations containing crospovidone and formulations containing combination of super disintegrants (CP + CCS, CP + SSG). This is

due to rapid uptake of the water from the medium, swelling and burst effect. It is also noticed that as the disintegrant concentration was increased from 4 to 8% the time taken for disintegration was reduced. The disintegration time of formulation (F3) containing 85% CP was found to be lower (10.30 ± 0.6) and was selected as the best ODT formulation among all the 18 formulations.

In vitro dissolution **:** The development of dissolution method for ODTs is almost similar to the approach taken for conventional tablets until they utilize the taste masking. The taste masking aspect greatly influences dissolution method development, specifications, and testing. Several factors like varied thickness and pH dependent solubility of drug particle coating influence dissolution profiles of ODTs containing taste masked actives. Since candesartan cilexetil is not bitter in taste, the bland taste of drug was masked by using sweeteners and flavors. It has been reported that USP type II apparatus with a paddle speed of 50 rpm is commonly used for ODT formulations. Slower paddle speeds are utilized to obtain good profiles as these formulations disintegrate rapidly.

In vitro dissolution studies of the prepared ODTs was performed in mixture of solvent pH 6.8 phosphate buffer and methanol (1:1) using USP dissolution apparatus type 2. The dissolution rate was found to increase linearly with increasing concentration of superdisintegrant. Formulations F1, F2 and F3 which contained increasing concentrations of Crosspovidone have recorded drug release 92.25 ± 0.15%, 97.42 ± 1.27% and 98.23 ± 0.02% respectively within 30 min. Formulations F4, F5 and F6 which contained increasing concentrations of Croscarmellose sodium have recorded drug release 94.8 ± 0.35%, 96.57 ± 0.45% and 92.17 ± 0.25% respectively, at the end of 30 min. Formulations F7, F8 and F9 which contained increasing concentrations of Sodium starch glycolate have recorded drug release 79.58 ± 0.13%, 85.92 ± 0.42% and 90.57 ± 0.48% respectively, at the end of 30 min.

Formulations F10, F11 and F12 which contained increasing concentrations of combination of CP + CCS have recorded drug release 98.27 ± 0.38%, 99.48 ± 0.34 % and 96.27 ± 0.16 % respectively, at the end of 30 min. Formulations F13, F14 and F15 which contained increasing concentrations of combination of CCS + SSG have recorded drug release92.91±0.38, 96.04±0.12, 97.68±0.06 at the end of 30 min. Formulations F16, F17 and F18 which contained increasing concentrations of combination of CP + SSG have recorded drug release respectively, 89.04 ± 0.43, 88.79 ± 0.12 and 94.28 ± 0.56at the end of 30 min. The FTIR studies revealed that there was no interaction between drug and excipients

Conclusion

Candesartan cilexetil Oral Disintegrating Tablets were prepared by direct compression method using Crospovidone, Croscarmellose sodium, Sodium Starch Glycolate and combinations of CP + CCS, CCS + SSG and CP + SSG as superdisintegrants exhibited good preformulation and tabletting properties. The above used superdisintegrants, formulation containing Crospovidone(F3) and combination of CP + CCS(F11) showed better performance in terms of disintegration time and drug release when compared to other formulations. Order of the superdisintegrant activity is as follows: (CP + CCS) > CP > CCS > (CCS+ SSG) > (SSG + CP) > SSG. candesartan cilexetil Oro Disintegrating Films were prepared by solvent casting method using different grades of Hydroxypropyl methylcellulose like HPMC – E15(P2), HPMC – 5 cps(Q2) and HPMC – 50cps(R3). Formulation Q2 exhibited faster disintegration time showing 99.98 ± 0.19 % drug release within 15 min. Based on disintegration and dissolution results, formulation Q2 was the best one from prepared ODTs and ODFs . Oral Disintegrating Tablets and Oral Disintegrating Films of candesartan cilexetil were found to improve the versatility, convenience, patient compliance leading to an enhanced approach for the administration of drug to the pediatrics and geriatrics.

Table 4. Composition of various formulation of ODT_s

Ingredients	Super disintegrants concentration (%) of Crosspovidone/ Croscarmellose Sodium/ Sodium Starch Glycolate		
	4%	6%	8%
candesartan cilexetil	4	4	4
Pearlitol SD 200	10	10	10
Avicel pH 101	59.2	57.6	56
Super disintegrants	3.2	4.8	6.4
Sodium saccharine (2%)	1.6	1.6	1.6
Orange flavor (2%)	1.6	1.6	1.6
Sodium stearyl fumarate (0.25%)	0.2	0.2	0.2
Talc (0.25%)	0.2	0.2	0.2

* All the amounts given in above table are in milligram

Table 5. Preformulation characteristics of candesartan cilexetil ODTs

Formulation	Bulk density (g/cc)	Tapped density (g/cc)	Hausner ratio	Compressibilty index (%)	Angle of repose
F1	0.391	0.463	1.18	15.55	27.54
F2	0.375	0.442	1.17	15.15	29
F3	0.379	0.446	1.17	15.02	27.42
F4	0.380	0.450	1.20	15.55	25.82
F5	0.392	0.466	1.18	15.87	27
F6	0.374	0.440	1.17	15.00	26.75
F7	0.371	0.441	1.18	15.87	25.82
F8	0.376	0.444	1.18	15.31	26.20
F9	0.370	0.438	1.18	15.52	28.35

Table 6. Preformulation characteristics of candesartan cilexetil ODTs prepared with combination of super disintegrants

Formulation	Bulk density (g/cc)	Tapped density (g/cc)	Hausner ratio	Compressibilty index (%)	Angle of repose
F10	0.376	0.442	1.17	14.93	29
F11	0.379	0.445	1.17	14.83	27.25
F12	0.382	0.452	1.18	15.48	28
F13	0.362	0.432	1.19	15.27	28.65
F14	0.375	0.445	1.18	15.73	27
F15	0.368	0.438	1.19	15.98	29
F16	0.372	0.440	1.18	15.45	26.54
F17	0.368	0.436	1.18	15.59	25.23
F18	0.382	0.452	1.18	15.48	24.87

Table 7. Tabletting characteristics of candesartan cilexetil ODTs

Formulation	Hardness[a] (kg/cm^2)	Friability[b] (%)	Weight [c] (mg)	Thickness [a] (mm)	Drug content [c] (%)	Disintegration time[a] (sec)
F1	2.88±0.26	0.15	79.73±0.49	3.83±0.07	99.5±0.5	20.16±0.75
F2	2.73±0.08	0.13	79.89±0.56	3.84±0.009	98.83±1.04	17.46±0.63
F3	2.8±0.18	0.32	79.85±0.58	3.84±0.01	101.48±0.5	10.3±0.6
F4	3.2±0.27	0.24	79.96±0.63	3.82±0.02	98.62±1.51	34.55±0.88
F5	2.96±0.15	0.27	79.83±0.84	3.82±0.01	97.59±0.52	29.71±0.77
F6	3.4±0.25	0.25	79.65±0.77	3.71±0.01	100.11±1.78	24.71±0.44
F7	3.2±0.40	0.19	79.90±0.90	3.77±0.02	98.18±0.86	30.05±0.64
F8	3.46±0.20	0.23	80.07±0.88	3.80±0.01	89.23±1.22	27.93±0.62
F9	3.5±0.15	0.25	80.13±0.87	3.72±0.02	101.05±1.58	19.40±0.80

Table 8. Tabletting characteristics of candesartan cilexetil ODTs prepared with combination of super disintegrants

Formulation	Hardness[a] (kg/cm^2)	Friability[b] (%)	Weight [c] (mg)	Thickness [a] (mm)	Drug content [c] (%)	Disintegration time[a] (sec)
F10	2.91±0.07	0.25	79.94±0.97	3.83±0.01	102.00±0.21	17.89±0.68
F11	3.1±0.21	0.34	80.02±0.90	3.84±0.005	96.5±0.5	13.5±0.83
F12	3.33±0.22	0.28	79.90±0.92	3.85±0.008	100.84±1.41	14.25±0.75
F13	3.1±0.22	0.54	79.95±0.79	3.83±0.005	99.81±1.08	24.90±0.92
F14	2.91±0.07	0.37	79.90±0.79	3.84±0.01	98.20±0.31	18.25±0.60
F15	3.35±0.15	0.27	80.02±0.82	3.85±0.005	87.41±0.72	14.16±0.75
F16	2.85±0.10	0.15	80.05±0.81	3.82±0.01	99.38±0.77	20.66±1.50
F17	3±0.06	0.37	79.87±0.79	3.83±0.008	99.65±1.42	20±0.89
F18	2.61±0.13	0.29	80.07±0.78	3.85±0.007	98.87±0.81	16.66±0.81

a: Mean ± S.D., n = 6 tablets, b: Mean ± S.D., n = 10 tablets, c: Mean ± S.D., n = 20 tablets.

References

1. Hisakadzu, S. and Yunxia (2002). Preparation, evaluation and optimization of rapidly disintegrating tablets. J. Powder Tech, 122: 188-198.

2. Puttewar, T.Y., Kshirsagar, M.D., Chandewar. A.V. and Chikhale.R.V. (2010). Formulation and evaluation of taste masked doxylamine succinate using ion exchange resin. J. king saud university, 22: 229-240.

3. Jeevanandham.S., Dhachnamoorthi.D., Chandrasekhr K.B., Muthukumaran.M., Sriram. N. and Joysarby. J. (2010). Formulation and evaluation of naproxen sodium orodispersible tablets - a sublimation technique. Asian J. pharm, 11: 49-51.

4. Jaysukh J H., Dhaval A .R. and Kantilal R .V. (2009). Orally Disintegrating Tablets: A Review, Tropical. J. Pharm. Res, 8 (2): 161-172.

5. Kuldeep .M., Kevin.G., Biswajit .B., Ravi. B.,Bhavik .J. and Narayana Charyulu.R. (2010). An emerging trend in oral drug delivery technology: Rapid disintegrating

tablets, J. Pharm. Sci. Tech, 2 (10): 318-329.

6. Sandeep .D., Kavitha.K. and Ganesh N.S. (2011). Fast disintegrating tablets – An emerging trend, Int. J. Pharm. Sci. Rev. Res, 6(2): 18-22.

7. Bhupendra G .P. and Ratnakar.N. (2009). A review on recent patents on fast dissolving drug delivery system, Int. J. PharmTech. Res, 1(3): 790-798.

8. Arun .A., Amrish .C., Vijay. S. and Kamla. P. (2009) .Fast dissolving oral films: an innovative drug delivery system and dosage form, Int. J. ChemTech. Res, 2(1): 576-583.

9. Abdelbary.A., Elshafeey.A.H. and Zidan.G. (2009).Comparative effects of different cellulosic-based directly compressed orodispersable tablets on oral bioavailability of famotidine, J. Carbohydrate Polymers, 77: 799-806.

10. Kulkarni A. S., Deokule H.A., Mane M.S. and Ghadge D. M. (2010). Exploration of different polymers for use in the formulation of oral fast dissolving strips, J. Cur. Pharm. Res, 2(1): 33-35.

11. Debjit .B.,Chiranjib.B., Krishnakanth P. and Margret Chandira.P. (2009) .Fast dissolving tablet: an overview, J. Chem. Pharm. Res, 1(1) :163-177.

12. Tejvir .K., Bhawandeep .G., Sandeep .K. and Gupta.G.D. (2011) .Mouth dissolving tablets: a novel approach to drug delivery, Int. J. Curr. Pharm. Res., 3(1):17.

13. Gavaskar.B., Subash Vijaya .k., Guru Sharan. and Madhusudan Rao.Y., (2010). Overview on fast dissolving films, Int. J. Pharmacy. Pharm. Sci., 2(3): 29-33.

14. Dixit.R.P. and Puthli.S.P.(2009) Oral strip technology: Overview and future potential. J. Contr. Release, 139: 94–107.

15. Hiroyoshi .S., Kazumi .T., Misao.N., Katsuhiko .M., Tadao. T., Hirotaka.Y., Naoki. I., Kazuyuki .H., Mayumi .Y., Yasutomi.K. and Yoshinori.I. (2009). Preparation of a fast dissolving oral thin film containing dexamethasone: A possible application to antiemesis during cancer chemotherapy, Eur. J. Pharm. Biopharm., 73: 361–365.

16. Kiran Bhaskar.D., More M. R., Sockan.G.N., Kunchu .K. and Mani.T. (2011). Formulation and evaluation of orodispersible tablets of propranolol hydrochloride, Int. J. pharm. Res. Dev, 2(12):46-52.

17. Siraj .S., Khirsagara.R.V. and Aamer .Q.(2010) . Fast disintegrating tablets: an overview of formulation and technology, Int. J. Pharmacy. Pharm. Sci, 2(3): 9-15.

18. Nehal S.Md., Garima .G. and Pramod Kumar .S. (2010)Fast dissolving tablets: preparation, characterization and evaluation: an overview, Int. J. Pharm. Sci. Rev. res, 4(2):87-96.

19. Rangasamy. M. (2009). Oral disintegrating tablets: a future compaction , Int. J. Pharma. Res. Dev. – online,1(10): 1-10.

20. Mohanachandran P.S.,Krishna Mohan.P.R., Fels Saju., Bini. K.B., Beenu .B. and Shalina K.K. (2010). Formulation and evaluation of mouth dispersible tablets of amlodipine Besylate, Int. J. Appl. Pharma, 2(3): 16.

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Survival and Apoptotic Signaling Pathways in Ovarian Follicle and Oocyte Development

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Abstract

Endocrine, cytokine and growth factors act in an autocrine and paracrine mechanisms for follicle and oocyte development. Current focus is on knowing how these activating and inhibiting factors act on primordial follicle to undergo transition from primary follicle to oocyte maturation. During oocyte development, due to LH stimulation, ligand of GPR3 is inactivated leading to inactivation of Gs subunit of GPR3 and in turn activation of Gi subunit of GPR3 by GTPase-activating proteins (GAPs). Gi which in turn inactivates Adenyl cyclase (AC) leads to meiotic resumption. Rise in Ca2+ level following LH stimulation also inhibits AC and activates phosphodiesterase (PDE) in turn leading to oocyte maturation. Apoptosis is essential for ovary function and development. Apoptosis loss includes quality control to eliminate meiotic anomalies. Only less than 1% of ovarian follicles which are present at birth will complete their path to ovulate. In fetal life oocyte generally undergo apoptosis, in adult life granulosa cell undergo apoptosis. These events are governed by pro-survival and pro-apoptotic factors. The equilibrium between these two factors (pro-survival molecules like Bcl-2, Bcl-x, TRAIL, TVB and the pro-apoptotic molecules like Bax, Bok, Bad, and Bak) may contribute to the regulation of ovarian follicle and oocyte apoptosis. Identification of these factors or patterns of expressions may contribute to the knowledge of mechanisms of growth of healthy oocytes with full developmental competence.

Key wowds: Pathways, apoptosis, follicle, oocyte, development, mammals

Introduction

Oogenesis starts before birth and continues through out reproductive life. Primordial germ cells from yolksac travels to genital ridge and undergoes several mitotic division forming primary oocyte and is arrested. After reaching puberty it undergoes first mieotic reductional division forming secondary oocyte, after fertilization secondary oocyte undergoes second mieotic equatorial division then formation of diploid zygote(1). All mammalian oocytes are arrested in ovary in first meiotic division and in prophase I of diplotene stage in primordial follicles. Folliculogenesis is the progression of a number of small primordial follicles to preantral, antral stage and then into large preovulatory follicles. The three stages of follicle development are: 1.Gonadotrophin independent 2. Gonadotrophin responsive 3. Gonadotrophin dependent (2, 3). Primordial follicles give rise to all dominant follicles. Arrested primordial follicle will enter into the pool of growing follicles is termed recruitment or primordial follicle activation. A primordial follicle contains a small primary oocyte arrested in the prophase I of diplotene stage of meiosis I with a single layer of flattened or squamous granulosa cells. In primordial follicle oocyte is surrounded by squamous shaped granulosa cells, after activation to primary follicle there is a change in shape of granulosa cell from squamous to

cuboidal. Acquisition of mitotic potential in the granulosa cells is a hallmark of recruitment and also there is growth of oocyte and differentiation, diameter of oocyte increases and development of its extracellular matrix, the zona pellucida (ZP).

***Activating Factors in ovarian follicle and ovum development*:** Transcription factors like basic helix-loop-helix (FIGLA), Forkhead box L2(Fox l2) (4), leukaemia inhibitory factor(LIF), kit ligand(KL), bone morphogenetic protein-15(BMP-15), BMP-4 (5), basic fibroblast growth factor(bFGF), keratinocyte growth factor(KGF) (6), BMP-7, platelet-derived growth factor(PDGF) (5). Oocyte specific genes are Nobox(newborn ovary homeobox encoding gene), Sohlh1, Sohlh2 and Lhx8 (LIM homoeodomain protein) (3, 5, 7), Glial derived neurotrophic factor(GDNF) and Neurotrophins like NGF, NTF5 and BDNF (8).

Inhibiting factors in ovarian follicle and ovum development: Factors include anti-Mullerian hormone (AMH), forkhead transcription factor O3(FOXO3) a downstream effector of the PTEN/ PI3K/AKT signaling pathway, a chemokine stromal cell derived factor(SDF-I) and its receptor(CXCR4) (9), phosphatase tenson homolog(PTEN), CXCL12, a Chemokine (7), inhibits ovarian follicle and ovum development.

PTEN-PI3K-Akt Signalling Pathway in Primordial follicle activation: The serine/ threonine kinase Akt (also known as protein kinase B or PKB) has critical regulatory role in diverse cellular processes. The Akt pathway is activated by receptor tyrosine kinases, integrins, B and T cell receptors, cytokine receptors, G-protein-coupled receptors and other stimuli that induce the production of phosphatidylinositol 3,4,5 triphosphates (PtdIns(3,4,5) P3) by phosphoinositide 3-kinase (PI3K). There are three highly related isoforms of Akt (Akt1, Akt2, and Akt3) and these represent the major signaling arm of PI3K. Akt regulates cell growth through its effects on the TSC1/TSC2 complex and mTOR pathways, as well as cell cycle and cell proliferation through its direct action on the CDK inhibitors p21 and p27, and its indirect effect on the levels of cyclin D1 and p53. Akt is a major mediator of cell survival through direct inhibition of pro-apoptotic signals such as the pro-apoptotic regulator Bad and the Fox0 and Myc family of transcription factors (http://www.cellsignal.com/ reference/pathway/pdfs/Akt_PKB).

Primordial follicle is arrested at the time of birth, after birth through PI3K-Akt signaling pathway it is activated and transferred to primary follicle. In arrested state FOXO3 is activated i.e, dephosphorylated form present inside nucleus which suppress primordial follicle activation. Upon binding of activating factors to Phosphatidyl inositol 3 hydroxy kinase (PI3K), present on oocyte membrane convert Phosphatidyl inositol diphosphate (PIP2) to Phosphatidyl inositol trihosphate (PIP3). PIP3 activates protein kinase b(Akt) by phosphorylating it, which inactivates Foxo3 by phosphorylation, Foxo3 moves out of nucleus leads to activation of primordial follicle activation. Inhibiting factors will activate PTEN thus primordial follicle will be in arrested state. PTEN inactivation is by binding of activating factors to oocyte membrane (10). Within oocytes, Foxo3 is regulated by nucleocytoplasmic shuttling. Foxo3 is imported into the nucleus during primordial follicle assembly, and is exported upon activation. Oocyte-specific ablation of PTEN resulted in PI3K-induced Akt activation, Foxo3 hyperphosphorylation, and Foxo3 nuclear export, thereby triggering primordial follicle activation, defining the steps by which the PI3K pathway and Foxo3 control this process. The principal physiologic role of the PI3K pathway is to control primordial follicle activation via Foxo3 (11). The improper activation of these two factors leads to premature oocyte activation and leads to rapid depletion of follicle reserve and leads to premature ovarian failure.

***Primary to secondary follicle transition*:** The development of a primary to a fully grown secondary follicle results from an active autocrine/ paracrine regulatory process that involves growth factors produced by the oocyte. Accumulation of increased numbers of granulosa

cells forms multiple layers around the oocyte. Granulosa cells develop to stratified or pseudostratified columnar epithelium from cuboidal epithelial cells. Two primary layers of theca appear; an inner theca interna and an outer externa appear. The activating factors are BMP-15, GDF-9, IGF-I (3, 12). At the time of follicle recruitment, GDF-9 and BMP-15 work in a co-operative function in regulating follicle cell proliferation, by GDF-9 during the early phases of folliculogenesis and by BMP-15 during more advanced phases (3). In antral follicles, KL factor up-regulates the expression of activin in granulosa and theca cells, which in turn positively regulates FSH secretion. FSH secretion is, in contrast, negatively regulated by both inhibin and follistatin. Insulin-like growth factor (IGF)-I has a role similar to that of activin, controlling FSH receptor expression in granulosa cells (3). IGF-I and FSHR are selectively co-expressed in healthy, growing follicles and that FSH does not affect IGF-I expression but IGF-I augments granulosa cell FSHR expression (12). Ovarian IGF-I expression serves to enhance granulosa cell FSH responsiveness by augmenting FSHR expression (12).

***Antral development*:** In antral development there are appearances of a fluid filled cavity at one pole of the oocyte and formation of Corona Radiata cells around oocyte. Mural granulosa cells are in basement membrane and antral granulosa cells are near antrum. During antral development as a consequence of FSH on cumulus cells, genes like high mitotic index, Insulin like growth factor1(IGF1), hyaluronic acid synthase2 (HAS2), PTX,TNFAIP-6, Cyclooxygenase (COX2) are expressed., on periantral granulosa cells versican and ADAMTS-1 are expressed, LH on theca cells express p450 gene. Both LH and FSH on mural granulosa cells express p450ssc, p450arom, Kit ligand and u-PA gene (2). The activating factors are FSH (13), GDF-9 (14), AMH, granulosa-derived kit ligand and oocyte gap junctional protein Connexin (Cx37) (2). The factors having role in entire folliculogenesis are TGFâ superfamily members (15, 16), Kit ligand (17, 18), activin, inhibin and follistatin (19).

***Signaling pathway during oocyte maturation*:** Oocyte maturation is progression from prophase I of meiosis I to metaphase of meiosis II, completion of meiosis II is only after fertilization. Primary and preantral follicles are mieotically incompetent. During oocyte maturation oocyte becomes mieotically competent. The fully grown oocyte with granulosa cells called cumulus oocyte complex (COC). By LH surge immature oocytes undergo meiotic resumption cytoplasmic modification and attain the fertilizable metaphase II (MII) stage. These events are regulated by pituitary horomones, steroids, growth factors (20). Before maturation oocyte will be arrested because of high levels of cAMP with in oocytes and or from surrounding cumulus cells through gap junctions. If cAMP is reduced with in oocyte then it will undergo meiotic resumption. The 5 factors which induces meiotic resumption are G protein coupled receptors, Gs- G stimulatory protein, G inhibitory proteins, Ca, cAMP phosphodiesterase (PDE) (21) which are acted upon by LH. Before LH stimulation GPR3 is active. Thereafter Gs is active and activation of adenyl cyclase leads to increase in cAMP that activates protein kinase A(PKA) which in turn act on cell cycle regulatory proteins i.e.,CDK1/Cyclin B(CYB) which gets phosphorylated and is inactive. This inactive form is called preMPK. cAMP phosphodiesterase is also low. Thus, cell cycle is arrested. By LH stimulation, it will block the ligand for G protein coupled receptor GPR3 and also inactivates G stimulatory protein (Gs) by GTPase enzyme, Gi is formed inactivates adenyl cyclase. Adenyl cyclase is also inactivated by rise in intracellular calcium by LH stimulation reducing cAMP level with in the cell. cAMP phosphodiesterase (PDE) is activated by LH stimulation leading to decrease in cAMP level in the cell, in active protein kinase, dephosphorylaytion of CDK1/Cyclin B(CYB) and active MPK cell cycle will continue , resumption of meiosis i.e., oocyte maturation.

***Apoptosis during follicle and oocyte development*:** In the ovary, the mechanisms underlying decisions of life and death involve balance between pro-apoptotic and pro-survival molecules. Apoptotic loss eliminate meiotic anomalies maintaining quality control, it's because of deficit in survival factors and a self-sacrifice phenomenon. In buffalo primordial follicle range from 10,000 to 19,000 and atresia is around 65-90%, while in cattle, the values are 150,000 and 65-70% respectively (22, 23, 24, 25). At least two-thirds of the oocytes present in the reserve degenerate by apoptosis before birth. Apoptosis is an essential component of ovarian function and development. During fetal life, apoptosis is found in the oocytes, in adult life in granulosa cells. Apoptosis involves two main phases of the meiotic process: an earlier one concerning the oogonia and oocytes in the preleptotene stage and a later one that concerns mainly the oocytes in the pachytene stage (26, 27).

***Apoptosis during fetal life*:** Germinal cells undergo apoptosis during gonadal mitosis and prophase of meiosis I. In primordial follicle formation atresia is thought to originate in the oocyte in primordial follicles. Once these follicles enter the growth pathway to become primary to antral follicles, apoptosis begins in the somatic granulosa cells, eventually leading to death of the oocyte and elimination of the follicle. The mitochondrial DNA is more liable to undergo mutations than genomic DNA and most of the mitochondria are inherited from the oocytes. Thus the quality of the mitochondria seems to have a crucial role in the life/death decision of the oocyte. Mitochondrion from healthy (non-apoptotic) granulosa cells are injected into the oocyte which decreased the possibility of apoptosis in these cells. After birth, most oocytes survive, and by puberty 75% loss in the ovarian follicles (28).

Apoptosis during adult life: The individual fate of a follicle (growth/ovulation *vs* atresia) is tightly regulated by a dialogue of death and survival signaling, including both endocrine factors (gonadotropins) and paracrine factors (e.g. GDF9, BPM15) (29). Only few primordial follicles present at birth progress to ovulation, the others die by apoptosis during the regulated process of follicular atresia.). Two types of follicular atresia are present (30): *a)* Basal atresia: It occurs throughout the reproductive life and its independent of estrus/menstruation cycle. There is premature involution of oocyte and low level of nuclear fragmentation is seen in the granulosa cells. Preantral follicles and follicles at the start of antrum undergo atresia in this stage. (31), b) Cyclic atresia*:* Larger follicles undergo atresia. Nuclear fragmentation is more in granulosa cells and degeneration of the oocyte (31). Normally one follicle will ovulate and rest of the follicle will undergo atresia. In primary follicle degeneration of oocyte then followed by granulosa cells. In secondary follicles degenerative changes start in ova and spread to follicular cells. In graafian follicle it starts with granulosa cells and then to the oocyte (31).

Survival factors: Survival factors regulating in ovarian apoptosis include peptides which are locally produced, cytokines, and steroid hormones. They are LH, P4, E2, IGF-1, EGF, bFGF, insulin, GH (31, 32, 33), Cyclic guanosine 3' 5'- monophosphate, IGF-1, Interleukin 1-β, EGF/TGFα, bFGF, Growth hormone (pitutary hormone), activin, nitric oxide, and estradiol, TGF A, bcl-2, bcl-xlong. (30, 32) Family of inhibitors of apoptosis proteins (IAPs) which will suppress apoptosis induced by different stimuli. 4 IAPs are present in mammalian ovary. NAIP-Neuronal apoptosis inhibitory protein XIAP-X-link inhibitor of apoptosis protein, HIAP-1 Human inhibitor of apoptosis protein-1, HIAP-2 Human inhibitor of apoptosis protein (30). Increase in expression with the development of follicle. Decrease expression was observed in the atretic follicles.

Atretic factors: Interleukin-6, Androgens, ROS, TNF-á (29), GnRH (31, 29), TGF-b, bax, Fas, p53 and caspases (33- 36, 30).

The prosurvival molecules (37) in ovary are:

Prosurvival Molecules	Mechanism of Action
Bcl-2	Apaf-1/caspases/Bax interactions
Bcl-x	Bcl-2/Mcl-1/Bax/c-Myc interactions
Mcl-1	Bcl-2/Mcl-1/Bax/c-Myc/Mcl-1/cytochromec interactions
Bcl-XL	Bcl-2/Mcl-1/Bax/c-Myc interactions
Boo	Mcl-1/Apaf-1/caspase9 interactions
TRAIL	TRAIL receptor interactions
TVB	Interaction with death receptor domain
GATA-6	GATA-6/gonadotrophin interaction
GATA-4	GATA-4/gonadotrophin interaction
C-kit	Interaction with SCF
SCF	C-Kit interaction
IAP	Interaction with caspase
Integrin	Interaction with other extracellular matrix proteins
XIAP	XIAP/FSH/NFkB interaction
Integrin-β6	Interaction with other extracellular matrix proteins
Integrin-β4	Interaction with other extracellular matrix proteins
Integrin-β	Interaction with other extracellular matrix proteins
NFκB	Caspase inhibitors
Survivin	Inhibits action of caspase
Gonadotrophins	BAX/Apaf-1/Fas/p53 interactions
Granzyme B	Allows apoptotic signal to bypass mitochondria
TGF-β 1	Interaction with smad/transcription factors
NAIPS	Interaction with gonadotrophins
TGF-β 2,3	Interaction with smad/transcription factors
TGF-β RII	Interaction with smad/transcription factors
GDF-9	Interaction with c-Kit/inhibin α
Smad 2,4	Interactions with transcriptional regulators
IGF	Interaction with IGF binding proteins
NTS	Interaction with TrκB kinase receptor
NOBOX	Interaction with GDF-9
AHR	Interaction with polycyclic aromatic hydrocarbon

The proapoptotic molecules (37) in ovary are:

Proapoptotic Molecules	Mechanism of Action
Bax	P53/Bcl-2/Bcl-XL/c-Myc interactions
Bok	Bok/cytochrome c interaction
Bad	Survival factor interaction
Bak	Boo-Apaf-1 disruption
Bik	Boo-Apaf-1 disruption
Caspases	Apaf-1 interaction
P53	Bcl-2/bax/cAMP interaction
Prohibitin	Mitochondrial destabilization
C-Myc	C-Myc/Bcl-2 interaction
Interferon	Interaction with Fas
Endothelins	Binding to two G-protein-coupled receptors
Par-4	Smad / transcription factors
Apaf-1	Cytochrome c/caspase interaction
Fas and FasL	Caspase interaction

Apoptotic Pathways: Apoptosis in ovary takes place by 2 pathways intrinsic/mitochondrial pathway and extrinsic pathway. Extrinsic pathway is through death receptors i.e., Fas, tumour necrosis factor receptor (TNFR), interferon (IFN) and TNF-related apoptosis-inducing Ligand (TRAIL) receptors (38). Death domains are present on the cell membrane when death receptors binds to its corresponding domain and cascades of events takes place. Extrinsic pathway is faster than intrinsic pathway. In intrinsic pathway or mitochondrial pathway the antiapoptotic molecules like BcL-2, BcL-X are present on outer mitochondrial membrane which prevents apoptosis by maintaining the mitochondrial membrane integrity (38, 35). When a cell receives endogenous negative signals like ROS lack of hormones pro-apoptotic molecule like Bax are released and damage the mitochondrial membrane integrity leads to leakage of Cytochrome C then cascades of apoptosis. Activation of caspases leads to apoptosome formation and formation of caspase 3 activates deoxyribonucleotidases causing fragmentation of cellular DNA and apoptosis (35, 37, 38). The anti-apoptotic Bcl-2 and Bcl-xL proteins inhibit cytochrome c release through the mitochondrial pore and also inhibit activation of the cytoplasmic caspase cascade by cytochrome c. Dephosphorylated BAD forms a heterodimer with Bcl-2 and Bcl-xL, inactivating them and thus allowing Bax/Bak-triggered apoptosis. When BAD is phosphorylated by Akt/protein kinase B (triggered by PIP_3), it forms the BAD-(14-3-3) protein homodimer. This leaves Bcl-2 free to inhibit Bax-triggered apoptosis. BAD phosphorylation is thus anti-apoptotic, and BAD dephosphorylation (e.g., by Ca^{2+}-stimulated Calcineurin) is pro-apoptotic.

Conclusions

During ovarian follicle development, the follicle growth is dependent and controlled by oocyte. Ovarian apoptosis takes place in the germ cells and granulosa cells. Identification and characterization of the factors contributing to the growth and developmental competence of oocytes can be used as markers of oocyte quality. The establishment of *in vitro* growth systems for ovarian follicles and oocytes may have many clinical applications. Increased understanding of the regulatory events of follicle and oocyte development will aid development of new contraceptives and new methods for increasing

the female reproductive lifespan and will lead to improvements in culture methods for oocytes for in vitro manipulation.

References

1. *Hafez*, E. S.E. B. and *Hafez*, B. (2000). Reproduction in farm animals, 7th Edition.
2. Campbell, B. K. (2009). The endocrine and local control of ovarian follicle development in the ewe**.** Anim. Reprod., 6: 159-171.
3. Zuccotti, M., Valeria, M., Sandra, C., Carlo, A. R. and Silvia, G. (2011). What does it take to make a developmentally competent mammalian egg? Human Reprod. Update., 17: 525–540.
4. Carmen, J.W. M.D. and Gregory, F.E. (2012). Morphology and Physiology of the Ovary. Chapter 1, Endocrinology of Female Reproduction, htttp://www.endotext.org/ female/ female1/ female1.htm
5. Nilsson, E. E. and Skinner, M. K. (2003). Bone Morphogenetic Protein-4 Acts as an Ovarian Follicle Survival Factor and Promotes Primordial Follicle Development. Biol. Reprod., 69: 1265–1272.
6. Kezele, P., Nilsson, E .E and Skinner, M. K. (2005). Keratinocyte Growth Factor Acts as a Mesenchymal Factor That Promotes Ovarian Primordial to Primary Follicle Transition, Biol. Reprod., 73: 967–973.
7. Pangas, S. A., Youngsok, C., Daniel, J. B., Yangu, Z., Heiner, W., Martin, M. M. and Aleksandar, R. (2006).Oogenesis requires germ cell-specific transcriptional regulators Sohlh1 and Lhx8, Dev. Biol., 103(21): 8090-8095.
8. McLaughlin, E.A. and Skye, C.M. (2009). Awakening the oocyte: controlling primordial follicle, Reproduction., 137: 1-11.
9. Holta, J. E., Andrew, J., Shaun, D. R., John, A. R., Peter, K. and McLaughlin, E. A. (2006). CXCR4/SDF1 interaction inhibits the primordial to primary follicle transition in the neonatal mouse ovary, Dev. Biol., 293: 449–460.
10. George, B., Teresa, D. G., Lane, J. S. and Diego, H. C. (2008). Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth, Dev. Biol., 321: 197–204.
11. John, G.B., Gallardo, T.D., Shirley, L.J. and Castrillon, D.H. (2008). Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth, Dev. Biol., 321:197-204.
12. Zhou, J., Rajendra Kumar, T., Martin, M. M. and Carolyn, B. (1997). Insulin-like growth factor regulates gonadotropin responsiveness in the murine ovary, Mol. Endocr., 11: 1924–1933.
13. Hsueh, A. J. W. and Billig, H. T. A. (1994). Ovarian follicle atresia: a hormonally controlled apoptotic process, Endocr. Rev., 15: 707–724.
14. Dong, J., Albertini, D. F., Nishimori, K., Kumar, T. R., Lu, N. and Matzuk, M. M. (1996). Growth differentiation factor-9 is required during early ovarian folliculo-genesis, Nature., 383: 531–535.
15. Knight, P.G. and Claire Glister. (2006). TGF-b superfamily members and ovarian follicle development, Reproduction.,132: 191-206.
16. Juengel, J. L. and Mc Natty, K. P. (2005). The role of proteins of the transforming growth factor-â superfamily in the intraovarian regulation of follicular Development, Human Reprod. Update., 11: 144–161.
17. Parrott, J. A. and Skinner, M. K. (1999). Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. Endocrinol., 140: 4262–4271.
18. Hutt, K. J., McLaughlin, E. A. and Holland, M. K. (2006). Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis, Mol. Human Reprod., 12: 61–69.

19. Knight, P. G. and Glister, C. (2001). Potential local regulatory functions of inhibins, activins and follistatin in the ovary, Reproduction., 121: 503–512.

20. Kimura, N., Hoshino, Y., Totsukawa, K. and Sato, E. (2007). Cellular and molecular events during oocyte maturation in mammals: molecules of cumulus-oocyte complex matrix and signalling pathways regulating meiotic progression, Soc Reprod. Fertil. Suppl., 63: 327-42.

21. Lisa, M. M. (2005). Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation, Reproduction., 130: 791–799

22. Erickson (1996). The ovary: Basic principles and concepts. In Felig P, Frohman L (eds): Endocrinology and Metabolism. 4th ed. New York: McGraw Hill.

23. Danell, B. (1987). Oestrous behaviour, ovarian morphology and cyclical variation in follicular system and endocrine pattern in water buffalo heifers, Sveriges Lantbruksuniversitet Uppsala., 54-94.

24. Grimes, R.W., Matton, P. and Ireland, J. J. (1987). A comparison of histological and non-histological indices of atresia and follicular function, Biol. Reprod., 37: 82-88.

25. Palta, P. (1998). Fertility augmentation by inhibin-based fecundity vaccines: potential applications in buffaloes, Buff. J. 14:1-19.

26. Alexander, B., Arkady, T., Howard, C. and Vladimir, T. (1999). The role of apoptosis in normal and abnormal embryonic development, Journal of Assisted Reproduction and Genetics., 16: 512-519.

27. John, A. R., Jock, K. F., Karla, J. H. and Jeff, B. K. (2011). Apoptosis in the germ line, Reproduction., 141: 139–150.

28. Perez, G. I., Maravei, D. V., Trbovich, A. M., Cidlowski, J. A., Tilly, J. L. and Hughes, F. M. (2000). Identification of potassium dependent and independent components of the apoptotic machinery in mouse ovarian germ cells and granulosa cells, Biology Of Reproduction., 63: 1358–1369.

29. Hussein, T. S., Thompson, J. G. and Gilchrist, R. B. (2006). Oocyte-secreted factors enhance oocyte developmental competence, Dev. Biol., 296: 514 521C.

30. Andreu, C. V. and Habibi, H. R. (2000). Factors controlling ovarian apoptosis, Annual Rev. Physiol. Pharmacol., 78: 1003-1012.

31. Elizabeth, A. M and Aaron J. W. Hsueh. (2000). Initial and Cyclic Recruitment of Ovarian Follicles, Endocr. Rev., 21(2): 200-214.

32. Kaipia, A. and Hsueh, A. J. W. (1997). Regulation of ovarian follicular atresia, Ann. Rev. Physiol., 59: 349-63.

33. Beyth, N. R., Chun, S. Y., Hsueh, A. J. W and Tsafriri, A. (1996). Early onset of deoxyribonucleic acid fragmentation during atresia of preovulatory ovarian follicles in rats, Biol. Reprod., 55: 1075–1080.

34. Chun, S. Y., Eisenhauer, K. M., Minami, S., Billig, H., Perlas, E. and Hsueh, A. J. W. (1996). Hormonal regulation of apoptosis in early antral follicles: follicle-stimulating hormone as a major survival factor, Endocrinology., 137:1447–1456.

35. Petculesc, C. L., (2012). Mechanisms of apoptosis in the ovary scientific Papers: Anim Sci. Biotech., 45: 188-192.

36. Levy, R., (2005). Apoptosis in oocyte, Gynaecology Obstrectis and Fertility., 33: 645-652.

37. Hussein, M.R., (2005). Apoptosis in the ovary: molecular mechanisms, Human Reprod Update., 11: 162–178.

38. Hussein, M. R., Haemel, A. K. and Wood, G. S. (2003). Apoptosis and melanoma: molecular mechanisms, J. Path., 199: 275–288.

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A Review on Non-Conventional Turmeric: *Curcuma caesia* Roxb

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Abstract

The traditional system of medicine involves the use of different plant extracts for treating various ailments. The use of medicinal plants as medicines is based on the folklore remedies used in different parts of the world. *Curcuma caesia* is lesser known species of the genus and has been used for centuries as folklore medicines. On the basis of pharmacological attributes of this plant as authenticated by various scientific studies, the rhizome is considered as potent source of medicine over the synthetic drugs. Recent advances in the research work in this plant and submission of the gene sequences in the databases has prompted us to compile this review. It deals about the plant and all the scientific studies carried out to introduce the plant and bring awareness about lesser known species to the scientific community. Presence of medicinally important bioactive compounds in *Curcuma caesia* depicts that it has great potential for becoming a future drug.

Keywords: Bioactive compounds, *Curcuma*, ethnomedicine, pharmacological, traditional,

Introduction

The use of medicinal plants for the treatment of diseases is associated with rich knowledge of plants and their products in different ethnic communities present in different parts of the world. Medicinal plants can be considered as the backbone of traditional medicine and are widely used to treat a plethora of acute and chronic diseases all over the world (1). They are known as “Chemical Goldmines” due to the presence of natural chemicals, which are acceptable to human physiological system (2). With the advent of modern medicine, herbal based medicine suffered a setback, but due to the multiple drug resistance, side effects with antibiotics, restriction in use of synthetic antioxidant drugs and limited availability of anticancer drugs has forced the scientist to search for new substances from plant origin (3). And so, nowadays, there is a revival of interest with herbal-based medicine and the herbal drug industries is now a very fast growing sector in the international market (4). India has one of the richest ethno-botanical traditions in the world with more than 7000 species of plants found in different agro ecosystems and used by various indigenous systems of medicine (2). India is one of the world’s twelve leading biodiversity centres with the presence of over 45,000 different plant species, out of this about 15,000 - 20,000 plants have good medicinal properties of which only about 7,000-7,500 are being used by traditional practitioners (3). The Siddha system of medicine uses around 600, Ayurveda 700, Unani 700 and modern medicine about 30 plants species (5). Over 95% of the plants used are collected from the wild sources, leading to real danger of extinction of many important medicinal plants, so there is urgent need to conserve these natural resources and bring awareness about the lesser known medicinal plants.

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The Genus *Curcuma*: The Zingiberaceae family constitutes a vital group of rhizomatous medicinal and aromatic plants characterised by the presence of volatile oils and oleoresins. The important genera coming under Zingiberaceae are *Curcuma, Kaempferia, Hedychium, Amomum, Zingiber, Alpinia, Elettaria* and *Costus* (2). The genus *Curcuma* has originated in the Indo-Malayan region (6). India has rich diversity of *Curcuma*, especially species and cultivar diversity (7). Out of the 100 or so species reported in this genus, about 40 are of Indian origin (8). The Genus *Curcuma* is gaining importance all over the world as a mighty cure to combat a variety of ailments, as it carries bioactive component credited with anti-inflammatory, hypocholestraemic, choleratic, antimicrobial, antirheumatic, antifibrotic, antivenomous, antiviral, antidiabetic, antihepatotoxic and anticancerous properties as well as insect repellent activity (9). Though *Curcuma longa* syn. (*C. domestica*) is the most commonly utilized species, other species such as *C. caesia, C. aromatica, C. amada ,C. kwangsiensis, C. zedoaria, , C. malabarica, C. angustifolia, C. montana, C. decipiens, C. alismaifolia, C. thorelii, C. comosa* etc. are also economically important. About 40 *Curcuma* species, 50 cultivars and 20 improved varieties of *Curcuma longa* and one improved variety of *Curcuma amada* are available in India (10).

***Curcuma caesia*:** The plant: *Curcuma caesia* Roxb*.,* commonly known as Black turmeric is a perennial herb with bluish-black rhizome. The rhizome of this plant is aromatic and yields an essential oil and has been traditionally used for centuries as a folklore remedy. The inner part of the rhizome emits a characteristic sweet smell, due to the presence of essential oil and so'Turkomans' use these tubers as a rubifacient to rub their bodies after Turkish bath (11). Northern tribes use Black Turmeric as a talisman to keep the evil spirits away, while in West Bengal it ûnds an important place in traditional system of medicine and is also used as a substitute for turmeric in fresh state, in Madhya Pradesh also the plant is regarded as very auspicious and it is stated that a person who possesses it will never experience shortage of cereals and food (12).This clearly states that this plant is not only important medicinally but also important socially as well as spiritually.

Synonyms of *Curcuma caesia* in Indian and foreign Languages

Language	Name
Hindi	Kali Haldi, Nar Kachura, Krishna kedar
Manipuri	Yaingang Amuba or yaimu
Marathi	Kala-haldi
Telugu	Nalla Pasupu, Manupasupu
Kannada	Kariarishina, Naru kachora
Bengali	Kala haldi
Mizo	Aihang, Ailaihang
Assamese	Kala haladhi
Arabic	Gadwâr aswad.
English	Black zedoary
French	Zédoaire noir
German	Schwarze zedoarwurzel
Italian	Zedoaria nera.
Malay	Temu hitam
Neplaese	Kaalo haledo
Turkish	Kara cadvar.

Significance of name: *Curcuma caesia* is vernacularly known as Kali Haldi (Black turmeric). The basic reason behind it is, as in West Bengal the rhizome of *Curcuma caesia* is used for worshipping Goddess Kali (Black), probably, for this reason it is popularly called as Kali Haldi in Hindi, inspite of the fact that in plant taxonomy, the word *caesia* means blue colour. In Mahabharata (The Epic of Hindus) Lord Krishna is depicted as blue skinned, hence the word Krishna can also mean blue colour by etymology so, *Curcuma caesia* is also called Krishna Kedara in Hindi since inside portion of the rhizome is blue coloured.

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Taxonomical classification

Superkingdom	Eukaryota
Kingdom	Viridiplantae
Phylum	Streptophyta
Unranked	Embryophyta
Unranked	Tracheophyta
Unranked	Spermatophyta
Division	Magnoliophyta
Class	Liliopsida
Order	Zingiberales
Family	Zingiberaceae
Genus	*Curcuma*
Species	*caesia*
Binomial name	*Curcuma caesia* Roxb. (13)

Habitat: *Curcuma caesia* is found in Java, India and Myanmar (14). Black Turmeric is native to North-East and Central India (15).It flourishes well in moist deciduous forest (16). It is also rarely found in Madhya Pradesh, Jharkhand, Chhattisgarh and Orissa (17).

Tribal practices: Even today, about 80% of the world's population relies on traditional plant based medicines for treating various ailments (18). *Curcuma caesia* plant is regarded as very auspicious and it is often used for magic remedies, tantric sadhana and medication by tribal people in various parts of India. In Chhattisgarh, the rhizome is used in dried powder form (Fig. 1 C) as folklore medicine for the treatment of wounds, cold, cough inflammation, leucoderma, tumors, piles, bronchitis, pneumonia, asthma rheumatic pains, tooth aches and infertility etc. Ethnomedicinal plants from Gohpur of Sonitpur district Assam was done and population used for the study was mainly dominated with assamese, Bodo, Mishing, Nepali and Santhal communities of local healers.They used fresh rhizome paste (50 g) mixed with *Musa balbisiana* fruit bark ash and applied once daily till cure in gout, sprains and bruises (19). Documentation of traditional herbal knowledge of Khamptis, a major tribe of the state of Arunachal Pradesh was done and found that they used crushed collar paste of *Curcuma caesia* plants to heal severe wounds and injuries (19). An ethnobotanical survey among the Garo tribal healers to gather information of the plants, ailments, formulations, and dosages were obtained from the tribal healers inhabiting the Madhupur region in Bangladesh and *Curcuma caesia* is used in inflammation of tonsils (20). Not only rhizomes but the roots of *Curcuma caesia* are used in Unani medicines for dyspepsia, stomach and liver tonic (21). In Manipur, traditionally the rhizome is used for treating leucoderma, asthma, tumors, piles, bronchitis etc. The paste is applied in bruises, contusions and rheumatic pains (22). The rhizome is often used by the Baiga, Sahariya, Agariya, Gond, Korku and other tribal people of Madhya Pradesh state of India for the treatment of pneumonia, cough, cold in children and for fever and asthma in adults (23).

Status of plant in India: The plant of *Curcuma caesia* is used in India for tantric sadhana and medication by tribal people, due to which it is overexploited and depleting faster. The National Medicinal Plant Board of India has listed 112 plants as crucially vulnerable species circulated by Ministry of Environment in 1997 and according to this, export of these species is not permitted without the permission of the legal competent authorities and this notification includes *Curcuma caesia* as well. This plant is in great demand in Central India and, due to indiscriminate exploitation the plant has been categorized as an endangered species (24).

Our state Chhattisgarh is known as "Herbal State" due to presence of wide varieties of medicinal plants. Floristic Study of the erstwhile seven district of Chhattisgarh was made by National Botanical Research Institute, Lucknow and identified 45 species as endangered taxa of the state and *Curcuma caesia* is also in this list. According to the Chhattisgarh State Medicinal Plant Board, 45 species including *Curcuma caesia* in the state has been identified as endangered species.

Cultivation and Harvesting Methodology: The cultivation and harvest practices are similar to

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that of *Curcuma longa*. The propagating part of the plant is underground short and thick rhizome. The crop is grown in warm and moist regions. The soil should be sandy or clay loam and rich in humus. It can be grown in up to an altitude of 4000 feet above sea level. Small pieces of rhizome with a bud are sown 3 inches deep into soil in the months between April to August. The turning yellow of leaves and their fall in December to January indicate maturity of the crop. The rhizomes are then dug out leaving a few which serve as a seed for the next cropping season (25).The present harvesting practices of *Curcuma caesia* is very deteriorating because of high price of product in the national and international markets, immature collection by locals, poor regeneration. This may be the reason for the present status of the plant (26).

Morphological Characters: Syamkumar and Sasikumar (27) characterized 15 economically important *Curcuma* species and listed their important morphological characters. *Curcuma caesia* and *C. aeruginosa* have almost similar morphological characters and sometimes *C. aeruginosa* is misidentified as *C. caesia*, the only difference lies in ploidy level which is 63, colour of corolla is white and rhizome is virdis green in case of *C. aeruginosa* while *C. caesia* is a perennial herb with ploidy level as 42 and with following morphology.

Floral Character: Spike position - Central or lateral, Colour of calyx and corolla - Purple.

Rhizome Character: Colour of rhizome - Blue (Fig. 1 A), Aroma - Camphoraceous, Taste - Bitter

Aerial Character: Colour of leaf sheath and leaf midrib - Purple brown (Fig. 1 B).

Other morphological characters as described by other researchers are described as follows: Rhizome large, sessile tubers bluish grey inside; leaves large, oblong with a broad purple-brown cloud down the mid-rib; petiole long green, glabrous beneath; inflorescence spike appearing before leaves; fertile bract greenish; coma bract tinge with pink; flower pale yellow with bright yellow throat; calyx translucent white; corolla red (14). Verma *et al*., (28) reported detailed HPTLC studies for quantitative evaluation of active marker component including morphological and histological parameters to establish the authenticity of *C. caesia* rhizome and to differentiate the drug from its other allied species. Biochemical Component: Curcumin, essential oil and oleoresin are the three most important biochemical components found in *Curcuma* spp. (10).

Essential oil : An earlier study showed that the rhizome oil of *C. caesia* contains 76.6% δ-camphor (29), but later Banerjee *et al*., (30) reported that rhizome oil of *C. caesia* contains 1, 8-cineole (9.06%), ocimene (15.66%), 1-ar-curcumene (14.84%), δ-camphor (18.88%), δ-linalool (20.42%), δ borneol (7%) and zingiberol (12.60%). Behura (31) described the chemical composition of essential oil in rhizome as α-

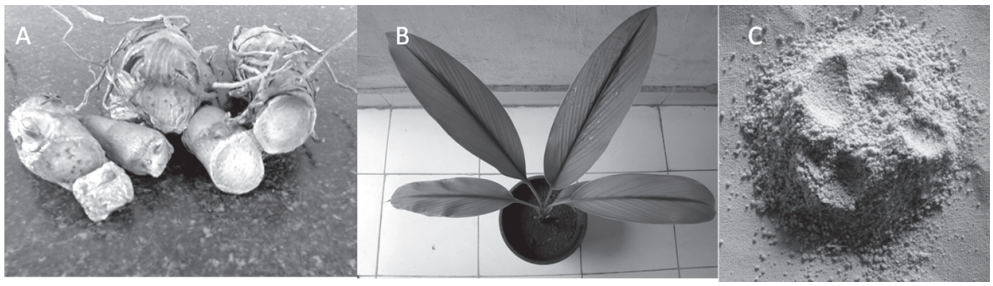

Fig. 1. Peculiar Morphological characters of *Curcuma caesia* Roxb., plant, which distinguishes it from other species of the genus. **A.** Characteristic blue rhizome of *Curcuma caesia*. **B.** Plant of *Curcuma caesia* with purple midrib in the leaf. **C.** Blackish grey coloured dried powder of rhizome used in folklore medicine.

Pinene (0.40), α-pinene (0.60), β-ocimene (E and Z) (2.1), camphor (7.73), linalool (0.99), caryophyllene (3.15), borneol (4.3), camphene (1.67), anethole (1.79) and cis-b-ocimene (14.54) Furthermore, the essential oil of the rhizomes of *C. caesia* from Thailand was characterized by a high content of 1,8-cineole (30.4%) and appreciable amounts of camphor (10.8%), curzerene (8.8%) and curzerenone (5.8%).This signiûcant variation in the camphor content of *C. caesia* and the search of new oil sources prompted for detailed GC– MS investigation of *C. caesia* (12). They found that volatile constituent of the rhizome oil from central India has major constituents as camphor (28.3%), ar-turmerone (12.3%), (Z)-β-ocimeme (8.2%), ar-curcumene (6.9%), 1,8-cineole (5.3%), β-elemene (4.8%), borneol (4.4%), bornyl acetate (3.3%), α-curcumene (2.8%), â caryophyllene (2.6%) and endo-fenchol (2.3%). They concluded that rhizome oil is deûcient in camphor, compared with the oil studied earlier. Furthermore paliwal *et al*., (23) reported that the Gas Chromato-graphy- mass spectrometry analysis of rhizome of Madhya Pradesh contains 1, 8 Cineole (27.48%), camphor (14-28.3%) as major constituent as well as ar-turmeone (12.3%). Behura and Shrivastava, 2004(32) also reported the essential oil content in the leaves as a-Pinene (1.5),b-pinene (6.3), myrcene (0.5), limonene (2.1), 1,8-cineole (27.0), camphor (1.68), linalool (2.8), b-elemene (2.4), borneol (8.7), a-terpenol (5.2) and eugenol (2.0).

Different authors have reported different profiles of essential oil found in *Curcuma caesia*. This anomaly may be due to environmental effects, varieties, maturity variations of the rhizome, analytical techniques and most important incorrect taxonomic identification of the specimens (10).

Bioactive component: The biological effects of turmeric is mainly attributed to its bioactive constituent, curcumin that has been widely used for its anti-inflammatory, antiangiogenic, antioxidant, wound healing and anti-cancer effects (33). In the rhizomes, curcumin and two related demethoxy compounds, demethoxy-curcumin and bisdemethoxy-curcumin, flavonoids and phenolic compounds which are widely distributed in plants have been reported to exert multiple biological effects. Sarangthem and Haokip (22) reported total curcuminoid as 78.4mg/g, volatile oil content as 6.75mg/g, total phenols as 60 mg/g, flavonoids as 30 mg/g, alkaloids as 104.25mg/g and soluble protein 47.5mg/g. This study showed that *Curcuma caesia* contain maximum curcuminoid , oil content, flavonoids, phenolics , different important amino acids, protein and high alkaloid content which reveals that the presence of these metabolites co-relates with its medicinal uses. A comparative study of phenol content and antioxidant activity of *Curcuma caesia* Roxb. and *Curcuma amada* Roxb. was done by Krishnaraj *et al*. (34), they reported that total phenol content and antioxidant activity were significantly high in *C. caesia* rhizome extract than the *C. amada* rhizome extract and although *C. amada* has already been introduced as a food additives, but there is no report available on the uses of *C. caesia* in food additives. Karmakar *et al*., (35) conducted study to evaluate the methanol extract of *C. caesia* rhizome for some *in vitro* antioxidant studies associated with reactive oxygen species and reactive nitrogen species and concluded that the methanol extract of *C. caesia* possesses good antioxidant activity which may be potentially responsible for its anti-inflammatory and chemoprotective mechanism as well as using this plant's extract as folkloric remedies. In *Curcuma caesia* alkaloids and tannins were found in addition to other metabolites while they were absent in *Curcuma longa.* In TLC profiling, the rhizome of both the *Curcuma* species showed presence of curcuminoids while in leaves only methanolic extract in *C. longa* showed distinctable bands for curcuminoids and in *C. caesia,* leaves showed only presence of curcumin while dis-methoxycurcumin and bis-demethoxycurcumin was found absent. Presence of medicinally important bioactive compounds in *Curcuma caesia* depicts that it has great potential for becoming a future drug for

treating various diseases (36).

Pharmacological attributes of the bioactive compound: Although *C. caesia* will have all the pharmacological attributes of *Curcuma* species due to presence of similar bioactive compounds but we have compiled only those research works which have been done in this species specifically.

The essential oils of *C. caesia* have been reported for both antibacterial and antifungal activities (37). Antifungal protein against *Candida albicans* has also been reported from *Curcuma caesia* Roxb. (Mannangatti and Narayanasamy) (38). *Curcuma caesia* is widely used in India as both an anti-inflammatory and anti-asthmatic in Ayurvedic medicine and preliminary mechanistic studies on the smooth muscle relaxant effect of hydro alcoholic extract of *Curcuma caesia* has also been reported (39). Neuropharmacological assessment of *Curcuma caesia* rhizome in experimental animal models was carried out to evaluate the methanol extract of *C. caesia* rhizome (MECC) and the study revealed remarkable analgesic, locomotor depressant, anticonvulsant and muscle relaxant effects of, demonstrating depressant action on the central nervous system and validated its traditional uses (40) Comparative evaluation of some plant extracts on bronchoconstriction in experimental animals was conducted, *Curcuma caesia* in 500 mg/kg showed significant protection against histamine induced bronchospasm (41).

Biological attributes of phytochemicals: Curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin, methyl curcumin and acetyl curcumin), volatile oil components from rhizomes and leaves of *Curcuma* species besides ethanol, crude ether form and water extracts of turmeric powder as well as powdered turmeric are very important biologically. In fact, the relative proportion of the different curcuminoids plays a considerable role in its bioprotective activity (10). The chemical structures of the biologically active compounds are:

Molecular Characterization in Curcuma caesia: Molecular studies in this species are in very primitive stage. Molecular genetic

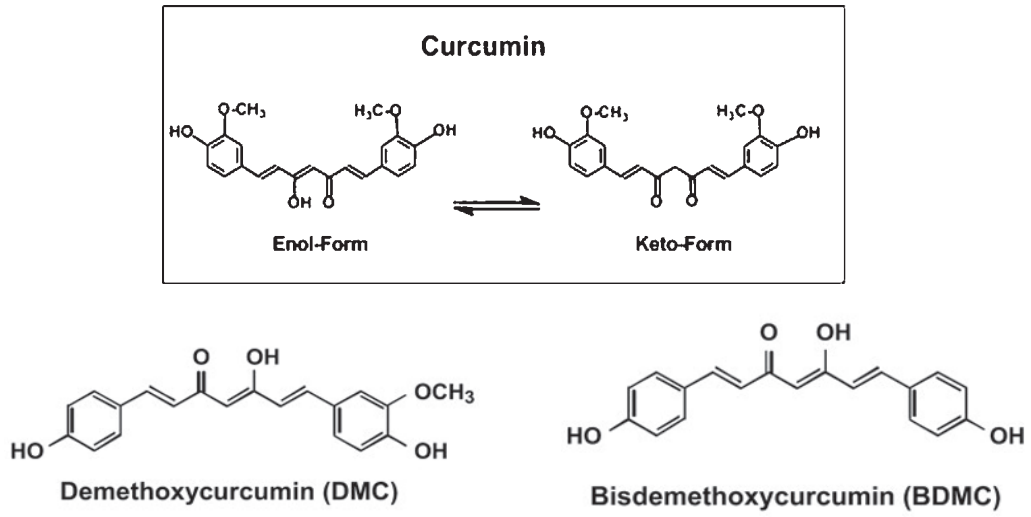

Fig. 2. Chemical structures of the pharmacological important curcuminoids.

ûngerprints of 15 *Curcuma* species including *Curcuma caesia* using Inter Simple Sequence Repeats (ISSR) and Randomly Ampliûed Polymorphic DNA (RAPD) markers to elucidate the genetic diversity among the species were formed. Thirty-nine RAPD primers yielded 376 bands of which 352 were polymorphic and out of the 91 bands produced by the 8 ISSR markers, 87 were polymorphic. Cluster analysis of data using UPGMA algorithm placed the 15 species into seven groups. *Curcuma caesia* and *C. aeruginosa* formed the fourth group in cluster II. The pairing of *Curcuma caesia* possessing deep blue coloured rhizome and camphoraceous aroma and bitter taste with *Curcuma aeruginosa*, having similar morphological and rhizome characters is very interesting. Between them they shared 72% similarity. Rhizomes of both the species are rich in camphor and their leaf midrib is having purple colour (42).

Intraspeciûc genetic diversity of four *Zingiber* species using Amplified Fragment Length Polymorphism (AFLP) markers was elucidated. *Curcuma angustifolia* was placed in a separate cluster I inferring to its wild nature while *Curcuma domestica II, Curcuma aromatica*, and *Curcuma caesia* were found to form a subgroup which shows a more precise discrimination among them. This can be explained that *Curcuma domestica II* and *Curcuma aromatica* are highlands species, possess geographic similarity and have a strong aroma, thus are found to be related. Dendrogram revealed that the species that are the derivatives of genetically similar type clustered more together (43).

Molecular genetic ûngerprints were formed of nine *Cucuma* species from Northeast India using markers with the aim of elucidating there intra and inter-speciûc genetic diversity, important for utilization, management, and conservation. 12 random ampliûed polymorphic DNA, Nineteen Inter simple sequences repeats, and four ampliûed fragment length polymorphism primers produced 266 polymorphic fragments. ISSR conûrmed maximum polymorphism of 98.55% whereas RAPD and AFLP showed 93.22% and 97.27%, respectively. The dendrograms based on three markers data were basically same with minor changes showing interspeciûc differences were more signiûcant compared to intra-varietal ones. In RAPD cluster analysis, *Curcuma caesia and C. zedoaria* formed cluster I. All other species were grouped into the cluster II along with cultivated species. Cluster II formed 3 subgroups where in *C.* spp. is placed with *Curcuma amada* in subgroup 1 showing that the wild species does not make an independent cluster. The adjoined group of cultivated varieties with wild ones suggests that they have been evolved in course of time. *Curcuma domestica I, II, and C. aromatica* have physiological similarity of strong aroma and were also sub-grouped together in the RAPD dendrogram having least genetic distance. Coinciding with the results of RAPD, the clusters based on ISSR analysis divides the *Curcuma* species at their genetic distances segregating them more precisely. ISSR analysis has placed *Curcuma caesia* separately whereas other species were placed together with the cultivated species in another cluster. *Curcuma amada, C. angustifolia*, and *C. zedoaria* were found to be genetically closer to each other and placed in a single group inferring their vegetative and topological similarity also. AFLP markers separated the three varieties of a single species (*Curcuma longa, C. domestica* I and II) in two subgroups depicting that intraspeciûc variations also exist (44).

Molecular marker-based study of genetic variations facilitates in the delineation of *Curcuma* species in dendrograms which are suggestive of an evolutionary pattern among *Curcuma*. The results provided phylogenetic relationship between cultivated and wild relatives of *Curcuma*. ISSR ûngerprinting opens new and interesting possibilities in the characterization of the *Curcuma* plants, which still awaits proper systematic identiûcation. Exploration and evaluation of diversity would be of great signiûcance for *in situ* conservation of important *Curcuma* species especially for their scientiûc and commercial programmes (44).

In vitro* studies in *Curcuma caesia: *Curcuma* is gaining importance globally as a potential source of drugs to combat a variety of ailments. Though earlier studies of *Curcuma* biotechnology were focused mainly on standardization of tissue culture protocols, recent attentions have been towards molecular biology aspects of the genus (45).

***In vitro* micropropagation:** One of the earliest attempts on micropropagation of turmeric in India was that of Nadgauda *et al*., (46). They produce plantlets from young vegetative buds (sprouts) obtained from rhizome of two *C. longa* varieties. But first report on *in vitro* regeneration in *C. caesia* was reported by Balachandran *et al*., (47), they used rhizome sprouted bud of *C. caesia, C. longa* and *C. aeruginosa* in MS medium. MS along with BAP (3.0 mg/l) were found to be the best media. They concluded that there were significant differences between the treatments in the rate of multiplication in all the species except *C. caesia*. Then Tyagi *et al*., (48) developed *in vitro* plant regeneration and genotype conservation in eight varieties of *Curcuma*. They found 11.2 µM or 22.2 µM BAP as the best concentration of harmone for shoot regeneration in *C. caesia*. Bharalee *et al*., (49) reported *In vitro* clonal propagation of *Curcuma caesia* Roxb. and *Curcuma zedoaria* Rosc. from rhizome bud explants and MS + BAP (4.0mg/l) + NAA (1.5mg/l) was found to be most suitable for multiple shoot regeneration in *C. caesia*. Mohanty *et al*., (17) used sprouted rhizome buds of *C. caesia* as explants on MS media with various combination of BAP, IAA, NAA and kinetin. The percentage of explants forming shoots was highest in MS + BAP (3mg/l) + IAA (0.5mg/l). A higher concentration of BAP was found to be inhibitory for shoot initiation and kinetin alone had no role in shoot multiplication.

***In vitro* Conservation:** Balachandran *et al*., (47) developed short term conservation of *In vitro* grown plants of *C caesia*. They used polypropylene caps for enclosure of the culture and they found that the plants remained green and alive for 7 months and multiplied after subculturing, while the cultures enclosed with cotton plugs dried within 2.5 months, the reason proposed was that there is slow water loss in tubes with polypropylene enclosures. Tyagi *et al*., (48) conserved *C. caesia* in vitro regenerated plants in 23.2µM kinetin supplemented in MS media and for about 323 days the plants remained alive and green.

In vitro microrhizome development, *In vitro* pollination, *In vitro* mutation as well as genetic transformation protocol has also been developed in *C. longa* but there is no report in *C. caesia.*

Genetic stability studies of micropropagated plants: The *In vitro* micropropagation technique is an alternative to conventional method of vegetative propagation for rapid clonal multiplication of the plants. The genetic stability of *in vitro* conserved plants is of utmost importance for commercial utilization of the technique for large scale production of true to type plants. The work regarding the extent of genetic stability of micro propagated *C. caesia* was done by Mohanty *et al*., (17). They reported that tissue culture raised plants of *C. caesia* could be conserved in vitro through subculturing in an interval of 4 months and the genetic stability of micropropagated plants was studied with interval of 6 months up to 30 months in culture using cytophotometric, RAPD, ISSR analysis. Cytophotometric analysis of 151 plants revealed a unimodal distribution of the DNA content with a peak corresponding to 4C nuclear DNA and RAPD as well as ISSR analysis showed monomorphic bands in all 73 regenerants, thus confirming genetic uniformity among *in vitro* grown somaclones of *C. caesia*.

Conclusion

Curcuma caesia is non-conventional, lesser known turmeric because of its limited bioavailability. The plant is an important medicinal plant used by folk people for various ailments and is considered very auspicious in Chhattisgarh and Madhya Pradesh, as they believe that, person who posses it will never have shortage of food, it has the same sacred value as Tulsi

have in common Indian houses. The plant has now gained the status of endangered species and is on the verge of extinction due to various unfavourable factors and overexploitation. Thus efforts should be made to conserve and work for the betterment of this plant species. On the basis of pharmacological attributes of this plant, the rhizome can be considered as good source of medicine over the synthetic drugs. Recent advances in the research work in this plant and submission of the gene sequences in the databases has prompted us to write this review. This is the review compiling all the research done in every aspect of the plant which will be helpful to the researchers who plan in future to work in this plant species.

References

1. Mazumdar, M.E.H. and Rahman, S. (2008). Pharmacological evaluation of Bangladeshi medicinal plants for antioxidant activity. Pharmaceutical Biology. 46: 704–709.

2. Joy, P.P., Thomas, J., Mathew, S. and Skaria, B.P. (1998). Zingiberaceous Medicinal and Aromatic Plants. Aromatic and Medicinal Plants Research Station; Odakkali, Asamannoor P.O., Kerala, India.

3. Kataky, A. and Handique, P.J. (2010). A brief overview on Andrographis paniculata (Burm. f) Nees. high valued medicinal plant: Boon over synthetic drugs. Asian Journal of Science and Technology. 6: 113-118.

4. Sharma, A., Shanker, C., Tyagi, L.K, Singh, M. and Rao Ch.V. (2008). Herbal Medicine for Market Potential in India: An Overview. Academic Journal of Plant Sciences. 1:26-36.

5. Chaudhri, R.D. (1996). Herbal Drugs Industry. Eastern Publisher, New Delhi, 1st Edn.

6. Purseglove, J.W. (1968).Tropical Crops: Monocotyledons.; London: Longman.

7. Sasikuma,r B., Krishnamoorthy, B., Johnson, K.G, Saji, K.V., Ravindran, P.N. and Peter, K.V. (1999). Biodiversity and conservation of major spices in India. Plant Genetic Resources Newsletter. 118: 19–26.

8. Velayudhan, K.C., Muralidharan, V.K., Amalraj, V.A., Gautam, P.L., Mandal, S. and Kumar, D. (1999). *Curcuma* Genetic Resources. Scientific Monograph. No. 4. New Delhi: National Bureau of Plant Genetic Resources

9. Chattopadhyay, I., Biswas, K., Bandyopadhyay, U. and Banerjee, R.K. (2004). Turmeric and curcumin: biological actions and medicinal applications. Curr. Sci. 87: 44–53.

10. Sasikumar, B. (2005). Genetic resources of *Curcuma*: diversity, characterization and utilization. Plant Genetic Resources. 3: 230–251.

11. Kirtikar, K.R. and Basu, R.D.(1987). Ind. Med. Plants.; IV: 2422.

12. Pandey, A.K. and Chowdhury, A.R. (2003). Volatile constituents of the rhizome oil of *Curcuma caesia* Roxb. from central India. Journal of Flavour and Fragrances. 18: 463–465.

13. NCBI Taxonomy browser: http:/www.ncbinlm.nih.gov/taxonomy browser.

14. Sharma, G.J., Chirangini, P. and Kishor, R. (2011). Gingers of Manipur: diversity and potentials as bioresource. Genet Resour Crop Evol. 58: 753–767.

15. Ravindran, P.N., Nirmal Babu, K. and Sivaraman K. (2007). Turmeric: The Genus Curcuma. CRC Press. p. 11.

16. Nandkarni, K.M. (1976). Indian Medical. Medica. Vol 1 Bombay:Popular prakashan,pp- 414

17. Mohanty, S., Joshi, R. K., Subudhi, E., Sahoo, S., Nayak, S. (2010). Assesment

of genetic stability of micropropagated *Curcuma caesia* through Cytophotometric and molecular analysis. Cytologia, 75: 73-81.

18. Sen, P., Dollo, M., Choudhary, M.D., Choudhary, D. (2008). Indian journal of Traditional Knowledge, 7: 438-442.
19. Saikia, B. (2006). Ethnomedicinal plants from Gohpur of sonitpur district, Assam. Indian journal of traditional Knowledge, 5 : 529-530
20. Mia, M.M., Kadir, M.F., Hossan, Rahmatullah M. (2009). Medicinal plants of the Garo tribe inhabiting the Madhupur forest region of Bangladesh. American-Eurasian Journal of Sustainable Agriculture, 3: 165-171.
21. Faisal, S., Zaidia, H., Yamadab, K., Kadowakia, M., Usmanghanic, K., Sugiyamab, T. (2009). Bactericidal activity of medicinal plants, employed for the treatment of gastrointestinal ailments, against Helicobacter pylori. Journal of Ethnopharmacology, 121: 286–291.
22. Sarangthem, K. and Haokip, M. J.(2010). Bioactive component in Curcuma caesia Roxb. Grown in Manipur. The Bioscan, 5: 113 – 115.
23. Paliwal, P., Pancholi, S.S., Patel, R K. (2011). Pharmacognostic Parameters for evaluation of the rhizomes of *Curcuma caesia*. J Adv Pharm Tech Res. 2: 56-61.
24. Kumar, S., Singh, J., Shah, N.C., Ranjan, V. (1998). Indian Medicinal Plants Facing Genetic Erosion. CIMAP: Lucknow P- 219.
25. Bendre, A. and Kumar, A. Economic Botany. (1990). Rastogi publication Meerut, 4 edition. P-86.
26. Mishra, M. (2000). Harvesting practices and management of two critically endangered medicinal plants in the natural forests of central India. Proceedings in the International seminar on "harvesting of non-wood forest products", Held at Menemen-Izmir (Turkey), 2-8 October 2000. Pp: 335-341.
27. Syamkumar, S. and Sasikumar, B. (2007). Molecular marker based genetic diversity analysis of *Curcuma* species from India. Scientia Horticulturae, 112: 235–241.
28. Verma, D., Srivastava, S., Singh, V. and Rawat, A.K.S. (2010). Pharmacognostic evaluation of *Curcuma caesia* Roxb. rhizome. Natural Product Sciences, 12: 107-110.
29. Dutt S. (1940). Indian Soap J. 6: 248–255. Chem. Abs
30. Banerjee, A.K., Kaul, V.K. and Nigam, S.S. (1984). Essenze Deriv Agrum. 54: 117–121.
31. Behura, S. (2000). Gas chromatographic evaluation of *Curcuma* essential oils. In: Ramana KV, Santhosh JE, Nirmal Babu K, Krishnamurthy KS and Kumar A (eds) Spices and Aromatic Plants - Challenges and Opportunities in the New Century. Proceedings of the Centennial Conference on Spices and Aromatic Plants. Calicut, Kerala: Indian Institute of Spices Research. pp. 291–292.
32. Behura, S. and Srivastava, V.K. (2004). Essential oils of leaves of Curcuma species. Journal of Essential Oil Research, 16: 109–110.
33. Jayaprakasha, G.K., Rao, L.J. and Sakariah, K.K. (2006). Antioxidant activities of curcumin, demethoxycurcumin and bisdemethoxy curcumin. Food Chem. 98: 720-724.
34. Krishnaraj, M., Manibhushanrao, K. and Mathivanan, N.(2010). A comparative study of phenol content and antioxidant activity between non-conventional *Curcuma caesia* Roxb. and *Curcuma amada* Roxb.

International Journal of Plant Production. 4169-174.

35. Karmakar I, Dolai N, Saha P, Sarkar N, Bala A, Haldar PK. (2011). Scavenging activity of *Curcuma caesia* rhizome against reactive oxygen and nitrogen species. Oriental Pharmacy and Experimental Medicine. DOI 10.1007/s13596-011-0030-6

36. Behar, N., Tiwari, K.L. and Jadhav, S.K. (2013). Comparative phytochemical screening of bioactive compounds in *Curcuma caesia* Roxb. and *Curcuma longa*. Research journal of medicinal plants. 7(2): 113-118.

37. Rahman, M.A. and Yusuf, M. (1996). Diversity, Ecology and Ethnobotany of the Zingiberaceae of Bangladesh. J Econ Taxon Bot Addl. Series. 12: 13-19.

38. Mannangatti, K. and Narayanasamy, M. (2008). Antifungal protein from a medicinal plant, *Curcuma caesia* Roxb. J Biotechnol. 136S- S90.

39. Arulmozhi, D.K., Sridhar, N., Veeranjaneyulu, A. and Arora, S.K. (2006). Preliminary mechanistic studies on the smooth muscle relaxant effect of hydroalcoholic extract of *Curcuma caesia.* Journal of Herbal Pharmacotherapy. 6: 117-24.

40. Karmakar, I., Saha, P., Sarkar, N., Bhattacharya, S. and Haldar, P.K. (2011). Neuropharmacological assessment of *Curcuma caesia* rhizome in experimental animal models. Oriental Pharmacy and Experimental Medicine.11:251-255.

41. Paliwal, P., Pancholi, S.S. and Patel, R.K. (2011). Comparative Evaluation of some plant extracts on bronchoconstriction in experimental animals. Asian Journal of Pharmacy and Life Science. 1: 52-57.

42. Syamkumar, S. and Sasikumar, B. (2007). Molecular marker based genetic diversity analysis of *Curcuma* species from India. Scientia Horticulturae, 112: 235–241.

43. Kavitha, P.G., Kiran, A.G., Dinesh, Raj R., Sabu, M. and Thomas, G. (2010). Ampliûed fragment length polymorphism analyses unravel a striking difference in the intraspeciûc genetic diversity of four species of genus *Zingiber* Boehm. from the Western Ghats, south India. Current Sciences. 98: 242–247.

44. Das, A., Kesari, V., Satyanarayana, V.M., Parida, A. and Rangan, L. (2011). Genetic Relationship of *Curcuma* Species from Northeast India Using PCR-Based Markers. Molecular Biotechnology. 49: 65 - 76.

45. Parthasarathy, V.A. and Sasikumar, B. (2006). Biotechnology of *Curcuma* CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resource. 20: 1- 9.

46. Nadgauda, R.S., Mascarenhas, A.F., Hendre, R.R. and Jagannathan, V. (1978). Rapid multiplication of turmeric (*C. longa* L.) plants by tissue culture. Indian Journal of Experimental Biology. 16: 120–122.

47. Balachandran, S.M., Bhat, S.R. and Chandel, K.P.S. (1990). *In vitro* clonal multiplication of turmeric (*Curcuma* spp.) and ginger (*Zingiber officinale* Rosc.). Plant Cell Reports. 8: 521–524.

48. Tyagi, R. K., Yusuf, A., Dua, P. and Agarwal, A. (2004). *In vitro* plant regeneration and genotype conservation of eight wild species of *Curcuma*. Biologia Plantarum. 48: 129-132.

49. Bharalee, R., Das, A. and Kalitha, M.C. (2005). In vitro clonal propagation of *Curcuma caesia* Roxb. and *Curcuma* zedoaria Roxb. from rhizome buds explants. Journal of Plant Biochemistry and Biotechnology. 14: 61–63.

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Cytogenetic Analysis of Dairy Animals in India: An update

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Abstract

Animal clinical Cytogenetics started during 1960s. The majority of laboratories in India and abroad developed and started cytogenetic screening of cattle and buffaloes during last 5 decades which was parallel to human Cytogenetics. These laboratories were created almost exclusively within academic research institutions with a focus on basic research worldwide. However, a Cytogenetic screening programme in India was launched by the apex body, the National Dairy Development Board responsible for dairy development and animal improvement programmes during 1990s. Reduced fertility and infertility are major concern in dairy animals in India which could be due to poor breeding, feeding and management. However, it could also be due to chromosomal aberrations. Chromosomal aberrations might be transmitted or spontaneous generated during mitotic or meiotic cell divisions. Therefore, complete eradication of chromosomal aberrations from the dairy animal population may not be possible. In view of this the regular chromosomal screening especially of breeding bulls at the early age ought to be done This practice will not only reduce the occurrence of chromosomal abnormalities in dairy animal population rather it will save the time and amount spent on rearing of abnormal animals. Various kind of structural and numerical chromosomal abnormalities have been reported in India which is very less as compared to reported elsewhere. The chromosomal aberrations are reported mainly by conventional methods (banding of chromosomes) by which it is difficult to detect microdeletion, micro-duplication, microshiting translocation of minute fragments of chromosomes. The new molecular technologies like probe based fluorescence in situ hybridization (FISH) and microarray may be applied to detect minor abnormalities so that they can be correlated with infertility problems.

Key words: Chromosomal abnormalities, Aneuploidy, Chimerism, Mosaicism, translocation.

Introduction

Animal Cytogenetics started during 1960s by realizing the advantages of human clinical Cytogenetics. The majority of laboratories in India and abroad developed and started cytogenetic screening of cattle and buffaloes during last 5 decades. These laboratories were created almost exclusively within academic research institutions with a focus on basic research worldwide. However, a Cytogenetic screening programme in India was launched by the apex body, the National Dairy Development Board responsible for dairy development and animal improvement programmes during 1990s. Chromosomes are present in all nucleated cells of the body or eukaryotes. Chromosomes are nucleoproteins of the cells which carry genetic materials (genes) from one to another generation. The number and shape and size of the chromosomes are different in different species of animals. In cattle, reverine buffaloes and humans they are 60, 50 and 46 in number

respectively. Chromosomes are divided in two categories; sex chromosomes which determine sex of an individual, and others exist as homologous pairs and are known as autosomes. Arranging homologues pairs of chromosomes as per their shape and size is called karyotyping. A photomicrographs of an individual's chromosomes arranged in a descending order is called Karyotype. Chromosomes are classified as per the location of their centromere; telocentric, acrocentric, submetacentric and metacentric. In dairy animals, chromosomes are usually acrocentric and submetacentric.

Some of very specialized chromosome banding techniques developed in human Cytogenetics (1, 2) allowed rapid progresses in the animal Cytogenetics. An international study group with the mandate of standardizing the karyotypes of most farm animal species including cattle, buffalo, was created in 1976 during the Reading Conference (3). The Reading standard formed on the basis of all subsequent nomenclature reports (4-10).

Reduced fertility / infertility are major concern in dairy animals (cattle and buffaloes) due to poor breeding, feeding and management practices. To little extent, chromosomal abnormalities also contribute for reproductive failure in animals. Cytogenetics has several applications in animal improvement and one of them is the detection of chromosomal abnormalities usually associated with reduced fertility, infertility, embryonic loss, fetal waste and internal or external genital malformation. Quite a few bulls reared for breeding cannot be used effectively due to chromosomal abnormalities related to fertility. Chromosomal defects can affect breedable animals in two ways; a) the animal having chromosomal defects like sex chromosome mosaicism, inversions, trisomy-X, XXY etc., can be infertile or sub-fertile and b) the animals particularly AI bulls having chromosomal defects like Robertsonian translocation, reciprocal translocation, sex chromosomal mosaicism etc can cause repeat breeding problem in females.

In India, it has become mandate to screen breeding bulls before they are inducted in semen collection to avoid spread of such aberrations and repeat breeding. It is always advisable that the animal soon after birth may be screened for chromosomal aberrations so that the time and money spent on rearing of defective breeding animals can be saved. The routine chromosomal screening, to an extent, has been eliminating such animals which could have otherwise created reproductive failure in a large population. Routine screening of chromosome can only eliminate the abnormal one but cannot totally eradicate chromosomal aberrations from animal population as these abnormalities are spontaneous generated mainly during cell cycles (mitosis and meiosis). The chromosomal preparation and various banding pattern from lymphocytes culture can help us in routine investigation (11,12). Chromosomal aberrations could be numerical or structural or both in an individual. Many chromosomal abnormalities (numerical / structural / both) have been reported in cattle and buffaloes in India, which are described as under:

Chimerism is very common in cattle (13) and sheep (14) but also observed in buffalo (15), goat (16) and pigs (17). When two or more cell populations (XX/XY) derived from heterosexual zygotes the condition is known as chimerism. Developmental of placental anastomoses during pregnancy between heterosexual twins causes altered sexual development of a female co-twin due to the action of male hormones produced by a male co-twin. The factors whether environmental or hereditary for the development of placental anastomoses, are not clear. Some reports based on single observation, suggest hereditary tendency to develop anastomoses between co-twins in sheep (18). The freemartin syndrome occurs in about 92% of heterosexual twins (19), which can be diagnosed by leukocyte sex chromosomes chimerism, XY/XX. Chimerism usually affects fertility of females (13,20) but males are not grossly affected (21,22). However, on the contrarily many reports were published on reduced fertility or infertility of chimeric bulls.

Reduced fertility was observed (23) and many bulls were culled due to no semen ejaculation, had a low sperm count or had a high incidence of abnormal spermatozoa. Besides, the 7 chimaeric bulls were culled because of poor reproductive performance and testicular degeneration. Similar performance was also observed during studies of semen qualities in 15 chimeric bulls (24). They analyzed semen which included volume of ejaculate (ml), motility of spermatozoa (%) and sperm concentration/ml, and observed significant differences with regard to the volume of ejaculate and highly significant differences with regard to the motility of spermatozoa and sperm concentration. These parameters were lower in the group of chimeric bulls. There ratio of sex chromosomes (XX or XY) in blood cells have not been consistent irrespective to sex of animals (25). Around 20 river buffaloes (18 females and 2 males) were reported chimeric (26). All female carriers were sterile. Similar observation was observed in 8 female buffaloes (15). Some of chimerism cases were also reported with other chromosomal anomalies gave rise to different reproductive problems, as in case of a Friesian calf with an elongated urethra and without a vulva was born twin to a dead bull calf. Blood cell chimaerism and Mosaicism (XY/XXY) was found in the skin tissue (27).

Several cases of chimerism have been reported in Indian cattle (22,28,29,30) which were mostly in fertile males but their fertility was not evaluated. A few cases of buffalo chimerism (50,XY/50,XX) were also reported in India (31,32). Besides, many cases of chimerism in cattle and buffaloes probably have not been reported. By observing the impact of chimerism worldwide, it is advisable to examine the quality of semen of chimeric bulls before they are considered for semen collection centres.

Chimerism in cattle (33, 34) and other ruminant (35) may also be detected by polymerase chain reaction (PCR) by identifying amelogenine genes (AMELX and AMELY) responsible for tooth development (36) present on both X and Y chromosomes. The AMELY allele of Y chromosome contains a 63-bp deletion in the fifth exon as compared to the AMELX allele on the X chromosome. A PCR technique based on amplification of the region of AMX/Y containing this deletion uses a single primer pair to amplify a 280-bp fragment from the X chromosome and a 217-bp fragment from the Y chromosome (37). Different PCR based protocols have been used for sex determination. Some of the existing protocols can identify specific sequence on Y-chromosome such as sex determination region Y (SRY) gene (38,39) and testis-specific protein Y-encoded (TSPY) gene (40) or repeated sequences (41). Cytogenetic studies of leukocyte chimerism was performed with the *fluorescent in situ hybridization (*FISH) technique by using probes painting sex chromosomes to distinguish cell lines present in animals (42,43,44).

Mosaicism occurs due to mitotic non-disjunction during development. The coexistence of two or more genetically distinct cell populations derived originally from a single zygote is known as mosaicism. Mosaics may arise at any stage of development, from the two-cell stage onward, or in any tissue which actively proliferates thereafter. Mosaicism could be developed due to sex chromosomes or autosomes and both. The phenomenon is commonly observed in many species of animals and plants. Mosaic somatic alterations are present in all multi-cellular organisms, but the physiological effects of low-level mosaicism are largely unknown. Most mosaic alterations remain undetectable with current analytical approaches, although the presence of such alterations is increasingly implicated as causative for disease. In humans, when chromosomal mosaicism arises during development, pregnancy outcome depends on which tissue, and how much of that tissue is abnormal. In theory, cases with a relatively high proportion of trisomic cells are more likely to be associated with an abnormal outcome than those with a low proportion of trisomic cells. If a majority of the cells are abnormal then human development is likely to be abnormal. If only a tiny fraction of some tissue were involved,

the aneuploidy would likely to have little effect on growth and development. Perhaps many people carry a tiny and completely unimportant abnormal cell line somewhere in their body. However, a very minor degree of mosaicism could still be important if a crucial tissue carries the abnormal cells. An abnormal chromosome change confined to one part of the brain could theoretically impair neurological function (45), for example mosaic Down syndrome can be associated with a less characteristic facial appearance and milder mental impairment than those with typical trisomy-21. Chromosomally abnormal cells may also arise with age and contribute to such health problems as the occurrence of cancer in human. However, most age-related chromosome changes are likely either eliminated due to poor cell growth or have no obvious harmful effect, for example, 45,X0 cells are increasingly common in female blood cells as they age, but appear to have no harmful effect. Similarly, the effect of mosaicism on fertility of animals varies due to degree of mosaicism. A rare case of sex chromosome mosaicism (60,XX/90,XXY) in Holstein were associated with an aplastic vulva, penis and clitoris agenesis, a male-like urethra located in a pseudoprepuce opening between the mammary complexes and a well developed M. rectipeninus (46). An another case of mosaicism (60,XX/60,XX, t(12 q;15 q),inv (6) found to be associated with low fertility in Holstein cow which delivered three calves during 11 years of age (47). There are no much more reports on mosaicism and their effects especially in cattle and buffaloes. Recent report (48) presented the Parent-of-Origin-based Detection (POD) method for chromosomal abnormality detection in trio-based SNP microarray data. The method and software provide a significant advancement in the ability to detect low-level mosaic abnormalities, thereby opening new avenues for research into the implications of mosaicism in pathogenic and non-pathogenic processes.

In India, Sex chromosome mosaicism (XY/XYY) reported in a Jersey crossbred bull (49) associated with reduced fertility, 60,XX/61,XXX in an infertile HF cow (50) and XXY/XY in Jersey crossbred young bull (51). However, a low degree of Y chromosome mosicism (sub-metacentric/acrocentric) was also reported in Holstein crossbred young male (52). Similar case of Y chromosome instability was reported in three crossbred cattle (53) but its clinical significance has not been documented. Cases of mosaicism in Indian buffaloes are not much documented except a few cases. A case of mosaicism (50,XX/51,XX) was reported in 8 year old buffalo having irregular breeding history (54). Their Cytogenetic investigations revealed additional 5^{th} chromosome (51,XX+5) in 22.67% cells. The autosomal mosaicism (50,XY/51,XY+4?) in 10% of the cells in a young bull which was phenotypically normal, was observed (51). A case of mosaicism was observed in a phenotypically normal young calf of Gir (*Bos indicus*) cattle which exhibited inconsistent number of chromosomes 60,XY/58,XY/59,XY/61,XY/61,XYY/61,XY+17/3n in 21 out of 111 metaphase spreads (18.2%) (55).

Sex chromosome aneuploidy occurs due to non-disjunction during meiosis or the early cleavage stages (56). The condition of sex chromosomal aneuploidy could be trisomy-X, XXY, XO and very rarely XYY which is observed in many species of animals including cattle and buffalo. However, the sex chromosome aneuploidy has deleterious effect on fertility as it was found associated with degradation of the seminiferous tubules (57), testicular hypoplasia (58) in bulls, abortion of six month male fetus (XYY) (59) and spontaneous abortions and neonatal losses in cattle (60). In one of testing surveys, 2 heifers with trisomy-X and 2 bull calves with XXY were found with serious abnormalities (61). River buffaloes with XO and Trisomy-X have been found to be associated with reproductive failure (26).

Many cases of XXY were reported in Indian cattle. A case of 61,XXY was reported (62) in an infertile Jersey crossbred bull which was ejaculating semen with more than 90% dead sperms. A Jersey calf with poor body development exhibited 61,XXY during routine cytogenetic investigation (63). Two Holstein crossbred calves

also exhibited 61,XXY (64,65). However, their fertility could not be estimated as they were young and culled from the breeding programmes. Some of cases of sex chromosomal aneuploidy (monosomy-X or XO) in buffaloes (66,67) associated with sterility and gonadal dysgenesis and trisomy-X with sterility (68) were reported in India.

Autosomal aneuploidy occurs due to non disjunction in autosomes. Autosomal aneuploidy typically alters the shape and proportions in characteristic ways. Plants tend to be somewhat more tolerant of aneuploidy than animals. Many still births caused by autosomal trisomy like 61,XY+27, 61,XY+?, 61,XY+21 were found in a cytogenetic survey of aborted fetuses (69). This indicates that animals with autosomal aneuploidy may not survive, as such cases are not reported in live animals. An autosomal aneuploidy in a still born Sahiwal zebu calf was also reported in India (70).

Most of anueploidies in animals were detected by conventional cytogenetic techniques whereas molecular diagnosis is being applied especially in prenatal diagnosis in humans. All molecular techniques are believed to be accurate and carry a low risk of misdiagnosis (71). DNA probes used in prenatal diagnosis of aneuploidy by FISH on interphase nuclei provide an initial rapid screen preceding the full cytogenetic evaluation (72). Multiplex PCR (73) and the PCR methods suitable for the detection of fetal aneuploidy. Slota et al. (74) used sex chromosome painting probe to identify XXY in 8-month old Polish Red breed.

Centric fusion was first observed by WRB Robertson in grasshoppers (75), hence popularly known as Robertsonian translocation. When two acrocentric chromosomes fuse at or around centromere, they produce such chromosomal aberration. Translocations are considered as structural as well as numerical aberration as centromere fusion to changes the structure of chromosome and resulting in reduction in numbers. It is also most common chromosomal rearrangement to occur during the karyotype evaluation of the Bovidae (76). The effect of translocation to animal breeding was realized four decades ago when Gutavsson (77) reported reduction in fertility of female carriers of 1;29 Centric fusion translocation (CFT) in Swedish Red cattle that encouraged investigators to extend their studies to various breeds. It has been found in various frequencies in about 60 different breeds of both *Bos taurus* and *Bos indicus* during 1964 to 1990 (78). Since then in various countries worldwide regular cytogenetic screening became mandatory to all the AI bulls before used for breeding programmes. Translocations are categorized in different types; i) *centric fusion translocation* in which the centromeres of two acrocentric chromosomes fuse to generate one large metacentric or submetacentric chromosome. The karyotype of an individual carrying a centric fusion has one less than the normal diploid number of chromosomes. ii) *Reciprocal translocation* occurs between two non-homologous chromosomes in which two chromosomes break and then exchange the fragments. Individuals carrying such abnormalities still have a balanced complement of chromosomes and generally have a normal phenotype, but with varying degrees of subnormal fertility. The subfertility is caused by problems in chromosome pairing and segregation during meiosis. Translocations can be balanced means an even exchange of material with no genetic information extra or missing, and ideally full functionality, or unbalanced where the exchange of chromosome materials are unequal resulting in extra or missing part of chromosome. Various kinds of translocations especially centric fusion translocation reported worldwide in various breeds of cattle and buffaloes.

In India, 1;29 translocation in Jersey crossbreds (79,80), 7;16 translocation (81) in Holstein crossbred, 16;20 translocation in Deoni zebu cattle breed (82) and unbalanced 1;9 translocation in a Gir (*Bos indicus*) bull calf (83) were reported. However a few cases of translocations were reported in Indian buffaloes.

Vijh et al. (84) reported a partial translocation 50,XY,t(3q-;6q+) in Murrah buffalo male but the effect of the abnormality on fertility was not described. One case of unusual translocation (XXY chromosome complement due to X;X-translocation) was also reported in Murrah buffaloes (85) which was associated with azoospermia (86).

New molecular based techniques to identify precise chromosomes involved in translocations are being practiced in humans and animals. Fluorescence in situ hybridization (FISH), with the use of locus-specific BAC probes, facilitated description of the translocation (87). A rare case of centic fission, rob (1p;23) in buffalo was identified with the help of molecular markers (88).

Structural chromosomal abnormalities include deletion, duplication, inversion, ring chromosome, terminal deletion, shifting, isochromosomes, chromatid gaps and breaks, fragile sites, variant chromosome, marker chromosome, etc. which are observed more in human than animals. Some of these abnormalities have been found associated with reproductive failure (89,90,91,92) and congenital anomalies (93).

Many structural aberrations are reported in Indian cattle and buffaloes with their significant association with reproductive failure. Variant chromosome 3 (3p+) in river buffalo males and females (94,95) were found associated with reduced fertility, presence of secondary constriction on 24 chromosome (24q+) in buffalo bull was found with abnormal sperms (96). Fragile sites in number of chromosomes (97,98) were found in a group of sub-fertile cattle and buffalo bulls. Spontaneous pericentric inversion in buffalo (99) was associated with reduced fertility. Chromosome fragmentations, pulverization, premature centromeric division (PCD), polyploidy (3n and 4n), endoreduplication, fragile site etc were also observed by many workers in their published (32,51,100) or unpublished work.

However, microdeletions, duplications, terminal deletion etc. may not be detected by conventional G- banding. Such abnormalities can be detected by molecular techniques (101). Chromosome Micro Array (CMA) technique which is being used in detection of minor chromosomal aberrations specially deletions or duplications syndromes, as well as the pericentromeric and subtelomeric regions, is boon to understand the roles played by segment of chromosome or locus of specific genes thereon in the development of disease in human. Chromosomal Micro Array can detect genomic errors for each of the disorders that are usually identified by karyotype analysis, including Down syndrome (102), trisomy 13, trisomy 18, sex chromosomal abnormalities, and other rare genetic disorders reported to be associated with mental retardation and/or physical problems (103). CMA is more sensitive than older methods of chromosomal analysis, and is able to detect abnormalities that would not have been identified by karyotype analysis. Though Karyotype testing currently is still considered the standard testing but microarray test may replace karyotyping in the near future as it has the capability to zoom in on regions of the chromosomes that are too small to visualize using a microscope, allowing for a higher resolution to determine if smaller regions of the chromosome are extra or missing.

Conclusions

Over the past 50 years, hundreds of scientific publications reporting original chromosomal abnormalities generally associated with clinical disorders (mainly fertility impairment) have been published. Most of chromosomal abnormalities observed in India were basically part of the routine investigation and part of small research studies carried out by the post graduate students in various institutions and universities. Therefore, the number of animals karyotyped in India is very less as compared to cattle and buffalo population. The chromosome screening should be performed at large scale even in indigenous cattle and buffaloes so that it can be compared with exotic cattle for their occurrences. Besides,

many of chromosomal screening findings are not published due to lack of confirmation of the chromosomal abnormalities by various banding patterns, different techniques and their association with infertility/ reduced fertility. It is advisable to screen especially all breeding bulls before inducted in Artificial Insemination programmes preferably at calf hood stage so that the time and money spent on rearing of abnormal bulls can be saved. Though the chromosomal aberrations in large animals are less reported but as far as possible the chromosomal abnormalities observed, and their fertility performance may be correlated. The aborted materials and stillborn calves must be utilized for the cytogenetic studies to know the possible reason of fetal waste. The new techniques based on molecular biology, FISH, chromosomal painting, microarray etc may be developed for routine cytogenetic screening of dairy animals.

References

1. Seabright, M. (1971). A rapid banding technique for human chromosomes. Lancet. 2:971–972.
2. Dutrillaux, B., Laurent, C., Couturier, J. and Lejeune, J. (1973). Coloration par l'acridine orange, de chromosomes préalablement traités par le 5 bromodéoxyuridine (BudR). C.R. Acad. Sci. (Paris). 276: 3179–3181.
3. Ford, C.E., Pollock, D.L. and Gustavsson, I. (1980). Proceedings of the First International Conference for the Standardisation of Banded Karyotypes of Domestic Animals. University of Reading Reading, England, 2nd–6th August 1976. Hereditas. 92: 145– 162.
4. Gustavsson, I. (1988). Standard karyotype of the domestic pig. Hereditas.109: 151–157.
5. ISCNDA. (1989). International System for Cytogenetic Nomenclature of Domestic Animals. Di Berardino D, Hayes H, Fries R, Long S (eds). Cytogenet Cell Genet. 53: 65-79.
6. ISCNDB. (2000). International System for Cytogenetic Nomenclature of Domestic Animals. Di Berardino D, Di Meo GP, Gallagher DS, Hayes H, Iannuzzi L (eds). Cytogenet Cell Genet. 92: 283–299.
7. Iannuzzi, L. (1996). G- and R-banded prometaphase karyotypes in cattle (Bos taurus L.). Chromosome Res. 4: 448–456.
8. Popescu, C.P. Long, S. Riggs, P., Womack, J., Schmutz, S., Fries, R. and Gallagher, D.S. (1996). Standardization of cattle karyotype: Report of the committee for the standardization of the cattle karyotype. Cytogenet Cell Genet. 74: 259–261.
9. Bowling, A.T., Breen, M., Chowdhary, B.P., Hirota, K. Lear, T., Millon, L.V., Ponce de Leon, F.A. and Raudsepp, T. and Stranzinger, G. (1997). International system for cytogenetic nomenclature of the domestic horse. Chromosome Res. 5: 433–443.
10. Ansari, H.A., Bosma, A.A., Broad, T.E., Bunch, T.D., Long, S.E., Maher, D.W., Pearce, P.D. and Popescu, C.P. (1999). Standard G-, Q-, and R-banded ideograms of the domestic sheep (Ovis aries): homology with cattle (Bos taurus). Report of the committee for the standardization of the sheep karyotype. Cytogenet Cell Genet. 87: 134–142.
11. Patel, R.K., Radhakrishna, U. and Khoda, V.K. (1995). A modified technique of GTG banding in cattle and buffaloes. The Nucleus. 38:37-39.
12. Patel, R. K. and Khoda, V.K. (1998). A rapid technique of CBG Banding for study of Cattle and buffalo chromosomes. Ind.Vet. J. 75: 837.
13. Peretti, V., Ciotola, S., Albarella, O., Paciello, C., Dario, V. and Iannuzzi, L. (2008). XX/XY chimerism in cattle: clinical and cytogenetic studies. Sex Dev. 2:24-30.

14. Wilkes, P.R., Munro, I.B. and Wijeratne, W.V. (1978). Studies on a sheep freemartin, The Vet. Record. 102:140–142.

15. Iannuzzi L., Di Meo, G.P., Perucatti, A., Ciotola, F., Incarnato, D, Di Palo R., Peretti, V., Campanile, G. and Zicarelli, L. (2005). Freemartinism in river buffalo: clinical and cytogenetic observations, Cytogenet. Genome Res.108:355-358.

16. Ilbery, P.L.T. and Williams, D. (1967). Evidence of the Freemartin Condition in the Goat. Cytogenetics. 6: 276-285.

17. Bruere, A.N., Fielden, E.D. and Hutchings, H. (1968). XX-XY mosaicism in lymphocyte cultures from a pig with freemartin characteristics. New Zealand Vet. J. 16:31-38.

18. Gill, J.J.B. and Davies, D.A.R. (1991). XX/XY chromosomal chimerism in infertile sheep of the Cambridge breed. Genet. Sel. Evol. 23:126-130.

19. Biggers, J.D. and Mcfeely, R.A. (1963). A Simple method for the display of chromosomes from cultures of white blood cells with special reference of the ox, Nature. 199:718-719.

20. Padula, A.M. (2005). The freemartin syndrome: an update. Anim Reprod Sci. 87:93-109.

21. Long, S.E. (1979). The fertility of bulls born twin to freemartins: a review. Vet Rec. 104:211-3.

22. Patel, R.K., Radhakrishna, U. and Khoda, V. K. (1997). Unusual Silent blood chimerism in Holstein Friesian bull. Ind. J. Anim. Sci. 67:152-153.

23. Dunn, H.O., McEntee, K., Hall, C.F., Johnson, R. H. and Stone, W.H. (1979). Cytogenetic and reproductive studies of bulls born co-twin with freemartins. J Reprod. Fert. 57: 21-30.

24. Rejduch, B., Kozubska-Sobocinska, A., Danielak-Czec, A. and Babicz, M. (2011). Semen quality evaluation of young bulls carrying leukocyte chimerism 60,XX /60,XY. Annales. 29:47-53.

25. Greene, W.A., Dunn, H.O. and Foote, R.H. (1977). Sex-chromosome ratios in cattle and their relationship to reproductive development in freemartins. Cytogenet Cell Genet. 18:97-105.

26. Di Meo, G.P., Perucatti, A., Di Palo, R., Iannuzzi, A., Ciotola, F., Peretti, V., Neglia, G., Campanile, G., Zicarelli, L. and Iannuzzi, L. (2008). Sex chromosome abnormalities and sterility in river buffalo. Cytogenet Genome Res. 120:127–131.

27. Dain, A.R. and Bridge, P.S. (1978). A chimaeric calf with XY/XXY mosaicism and intersexuality. J Reprod Fertil. 54:197-201.

28. Balakrishna, C.R., Yadav, B. R. and Bhatti, A.A. (1979). Unusual chromosomes constitution of a bovine freemartin, Ind. J. Dairy Sci. 32:191-93.

29. Patel, R.K. and Radhakrishna, U. (1994). Cytogenetic studies on ET born heterosexual twins. ET Update. 3:11.

30. Patel, R.K. and Khoda, V.K. (1997). Numerical Chromosomal Aberrations in Chimeric HF bull. The Nucleus. 40:124-127.

31. Balakrishnan, C. R., Yadav, B.R., Sharma, P.A. and Goswami, S.L. (1981). Sex chromosome chimerism in heterosexual Murrah buffalo triplets. Vet. Record. 109: 112.

32. Katragadda, S., Patel, R.K. and Kotikalapudi, R. (2013). Blood cell Chimerism (XX/XY) in Murrah buffalo bulls. Bangladesh J. Anim. Sci. 42: 20-22.

33. McNiel, E.A., Madrill, N.J., Treeful, A.E., Buoen, L.C. and Alvin Weber, F. (2006). Comparison of cytogenetics and

polymerase chain reaction based detection of the amelogenin gene polymorphism for the diagnosis of freemartinism in cattle. J Vet Diagn Invest. 18:469–472.

34. Divar, M.R., Sharifiyazdi, H. and Kafi, M. (2012). Application of polymerase chain reaction for fetal gender determination using cervical mucous secretions in the cow. Vet. Res. Commun. 36: 215-220.

35. Pajares, G., Alvares, I., Fernandez, I., Perez-Pardal L., Goyache, F., Luis, J. and Royo, L.J. (2007). A sexing protocol for wild ruminants based on PCR amplification of amelogenin genes AMELX and AMELY. Arch. Tierz., Dummerstorf. 50: 442-446.

36. Sasaki, S. and Shimokawa, H. (1995). The amelogenin gene. Int J Dev Biol. 39:127-33.

37. Ennis, S. and Gallagher, T. F. (1994). A PCR-based sex-determination assay in cattle based on the bovine amelogenin locus. Anim. Genet. 25:425–427.

38. Takahashi, M., Masuda, R, Uno, H., Yokoyama, M., Suzuki, M., Yoshida, M. C. and Ohtaishi, N. (1998). Sexing of carcass remains of the Sika deer (Cervus Nippon) using PCR amplification of the SRY gene. Vet. Med. Sci. 60:713-716.

39. Mara, L., Pilichi, S., Sanna, A., Accardo, C., Chessa, B., Chessa, F., Dattena, M., Bomboi, G. and Cappai, P. (2004). Sexing of in vitro produced ovine embryos by duplex PCR. Mol. Reprod. Dev. 69:35-42.

40. Lemos, D.C., Lopes Rios, A. F., Caetano, L.C., Lobo, B. R., Vila, R.A., Martelli, L., Takeuchi, P.L. and Ramos, E.S. (2005). Use of TSPY gene for sexing cattle. Mol Biol. 28:509-514.

41. Kageyama, S., Yoshida, L., Kawakur,a K. and Chikuni, K. (2004). A novel repeated sequence located on the bovine Y chromosome: its application to rapid and precise embryo sexing by PCR. J Vet Med Sci. 665:509-514.

42. Kozubska-Sobocinska, A., Slota, E. and Pienkowska, A. (2003).The application of FISH technique for diagnosing of leukocyte chimerism in sheep (in Polish), Medycyna Weterynaryjna. 59: 987–989.

43. Rejduch, B., Kozubska-Sobocinska, A., Radko A., Rychlik T. and Slota E. (2004). The application of genetic markers for cell chimerism diagnosis in lambs. J. Anim. Breed. and Genet. 121: 1–7.

44. Rychlik, T., Kozubska-Sobocinska, A., Rejduch, B. and Sikora, J. (2005). The phenomenon of cell chimerism in goats Vet. Med. 50: 311–314.

45. Gardner, R.J.M. and Sutherland, G.R. (1996). Chromosome Abnormalities and Genetic Counseling. 2nd edn. Oxford, Oxford University Press.

46. Meinecke, B., Drogemuller, C., Kuiper, H., Burstel, D., Wohlsein, P., Ebeling, S., Wehrend, A. and Meinecke-Tillmann, S. (2007). A Diploid-Triploid (60, XX/90, XXY) Intersex in a Holstein Heifer.Sex Dev.1:59-65.

47. Roldan, E.R.S., Marani, M.S. and Lawzewitsch, I.V. (1984). Two abnormal chromosomes found in one cell line of a mosaic cow with low fertility. Gent. Sel. Evol. 16:135-142.

48. Baugher, J.D., Baugher, B.D. and Shirley, M.D. (2013). Pevsner J. Sensitive and specific detection of mosaic chromosomal abnormalities using the Parent-of-Origin-based Detection (POD) method. BMC Genomics. 14:367-389.

49. Patel, R.K. and Patel, S.M. (1999). A case of Mosaicism (60,XY/61,XYY) in a Jersey crossbred bull. Ind. J Vet. Res. 8: 50-53.

50. Patel, R.K. (2003). Sex chromosome mosaicism (60,XX/61,XXX) in an infertile HF heifer. Ind J Anim Reprod. 24:161-162.

51. Patel, V.A., Patel, R.K., Shah, P.B. and Parikh, P.A. (2011). Cytogenetic studies of

the dairy bulls. Wayamba J Anim. Sci. Article No. 1320750403.

52. Patel, R.K. (2005). Y chromosome mosaicism in a crossbred calf. The Nucleus. 48:77-79.

53. Balakrishnan, C.R. and Yadav, B.R. (1987). Instability of Y chromosome in crossbred males of cattle. NDRI Diamond Jubilee Commemorative. 3:424-430.

54. Yadav, B.R., Kumar, S., Tomer, O.S. and Balakrishnan, C.R. (1991). Mosaicism of 50,XX/51,XX in a Murrah buffalo Bubalus bubalis. Genet. Sel. Evol. 23:443-448.

55. Kotikalapudi, R. and Patel, R.K. (2013). A case of mosaicism in Gir cattle (Bos indicus). JPBAS. 1:128-132.

56. Hamerton, J.L. (1971). Human Cytogenetics. [Academic Press, Vol 1].

57. Molteni L., De Giovanni Macchi, A., Meggiolaro, D., Sironi, G., Enice, F. and Popescu, P.(1999). New cases of XXY constitution in cattle. Anim Reprod Sci. 55:107-113.

58. Dunn, H.O., Lein, D.H. and McEntee, K. (1980). Testicular hypoplasia in a Hereford bull with 61,XXY karyotype: the bovine counterpart of human Klinefelter's syndrome. Cornell. Vet. 70:137-46.

59. Schmutz, S.M., Coates, J.W. and Rousseaux, C.G. (1987). Chromosomal Trisomy in an Anomalous Bovine Fetus. Can Vet J. 28:61-62.

60. Schmutz, S.M., Moker, J.S., Clark, E.G. and Orr, J.P. (1996). Chromosomal aneuploidy associated with spontaneous abortions and neonatal losses in cattle. J. Vet. Diagn. Invest. 8:91-95.

61. Citek, J., Rubes, J. and Hajkova, J. (2009). Robertsonian translocations, chimerism, and aneuploidy in cattle. J Dairy Sci. 92:3481–3483.

62. Patel, R.K. and Patel, S.M. (2000). A 61,XXY chromosome complement in subfertile Jersey crossbred bull. Ind. J. Anim Reprod. 21: 68-69.

63. Patel, R.K. (2002). Sex chromosomal aneuploidy (61,XXY) in a Jersey calf. Ind J Vet Res. 11: 21-23.

64. Patel, R.K., Singh, K.M. and Soni, K.J. (2005). Sex chromosomal aneuploidy (61,XXY) in Holstein Friesian and Kankrej crossbred calf: a case report. Haryana Veterin. 49: 75-76.

65. Chauhan, J.B., Patel, R.K., Singh, K.M. and Soni, K.J. (2008). Identification of late replicating X- chromosome in a HF crossbred calf with sex chromosome aneuploidy (61,XXY). The Nucleus. 51: 51-53.

66. Prakash, B., Balain, D.S. and Lathwal, S.S. (1992). A 49,X0 sterile Murrah buffalo (Bubalus bubalis). Vet Rec. 130:559–560.

67. Yadav, B.R., Kumar, P., Tomer, O.S., Kumar, S. and Balain, D.S. (1990). Monosomy X and gonadal dysgenesis in a buffalo heifer (Bubalus bubalis). Theriogenology; 34:99–105.

68. Prakash, B., Balain, D.S., Lathwal, S.S. and Malik, R.K. (1994). Trisomy- X in a sterile river buffalo. Vet Rec. 134:241–242.

69. Coates, J.W., Schmutz, S.M. and Rousseaux, C.G. (1987). A Survey of Malformed Aborted Bovine Fetuses, Stillbirths and Nonviable Neonates for Abnormal Karyotypes. Can J Vet Res. 52: 258-263.

70. Yadav, B.R. (2000). Autosomal trisomy in zebu calf (Sahiwal breed of cattle). J. Cytogen. and Genet. 71:76.

71. Dudarewicz, L., Holzgreve, W., Jeziorowska, A., Jakubowski, L., and Zimmermann, B. (2005). Molecular methods for rapid detection of aneuploidy. J. Appl. Genet. 46: 207-215.

72. Ward, B.E., Gersen, S.L., Carelli, M.P., McGuire, N.M., Dackowski, W.R., Weinstein, M., Sandlin, C., Warren, R. and Klinger, K.W. (1993). Rapid prenatal diagnosis of chromosomal aneuploidies by fluorescence in situ hybridization: clinical experience with 4,500 specimens. Am. J. Hum. Genet. 52: 854–865.

73. Schouten, J.P., McElgun, C.J., Waaijer, R., Zwijnenburg, D., Diepvens, P. and Pals, G. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucl. Ac. Res. 30: e57.

74. Slota, E., Kozubska-Sobocinska, A., Kooecielny, M., Danielak-Czech, B. and Rejduch, B. (2003). Detection of the XXY trisomy in a bull by using sex chromosome painting probes. J. Appl. Genet. 44: 379-382.

75. Robertson, W.R.B. (1916). Chromosome studies. I. Taxonomic relationship shown in the chromosomes of Tattigidae and Acrididae, Locustidae and Gryllidae: chromosomes and variation. J. Morph. 27:179-331.

76. Wurster, D.H. and Benirschke, K. (1968). Chromosome studies in the superfamily Bovidae. Chromosoma. 25:152-171.

77. Gustavsson, I. (1979). Distribution and effects of the 1/29 Robertsonian translocation in cattle. J Dairy Sci. 62:825-855.

78. Popescu, C.P. and Pech, A. (1991). Une bibliographie sur la tranlocation 1/29 de bovines dans le monde (1964-1990). Ann Zootech. 40:271-305.

79. Thiagarajan, V., Rajasekaran, J. and Natarajan, N. (1990). Robertsonian translocation in an anoestrus heifer. Cheiron. 19:7-9.

80. Chauhan, J.B., Patel, R.K., Singh, K.M. and Soni, K.J. (2009). A dicentric Robertsonian translocation, rob (1;29) in Indian Jersey crossbred (Bos taurus x Bos indicus) bull. The Nucleus. 52:119–123.

81. Patel, R.K. (1999). A new case of Robertsonian translocation, rob(7;16) in HF crossbred Bull. Ind. J. Dairy Sci. 52:324-329.

82. Padeeri, M., Reddy, C., Jain, N., Reddy, S.K. and Patel, R.K. (2011). A Case of Centric Fusion Translocation in a Deoni (Bos indicus) Indian cattle bull calf. IIOAB J. 2:21-23.

83. Padeeri, M, Reddy, C., Jain, N., Reddy, S.K. and Patel, R.K. (2011). Identification of unbalanced 1;9 autosomal translocation in a Gir (Bos indicus) bull calf. The Ind. Cow J. 27:62-65.

84. Vijh, R.K., Tanita, M.S. and Sahai, R. (1994). Translocation in Murrah buffalo. Ind. J Anim. Sci. 64:534-538.

85. Patel, R.K., Singh, K.M., Soni, K.J. and Chauhan, J.B.(2006). Novel Cytogenetic finding: Unusual translocations X; X in Mehsana buffalo (Bubalus bubalis). Cytogen. and Genom. Res. 115:186-188.

86. Chauhan, J.B., Patel, R.K., Singh, K.M. and Soni, K.J. (2009). Impact of a novel Cytogenetic finding (unusual X;X translocation) on fertility of a buffalo bull (Bubalus bubalis). Buffalo Bulletin. 28:151-153.

87. Switonski, M., Szczerbal, I., Krumrych, W., and Nowacka-Woszuk, J. (2011). A case of Y-autosome reciprocal translocation in a Holstein-Friesian bull. Cytogenet. Genome Res. 132:22-25.

88. Di Meo G.P., Perucatti A., Genualdo V., Iannuzzi A., Sarubbi F., Caputi-Jambrenghi A., Incarnato A., Peretti V., Vonghia G., and Iannuzzi L., A Rare Case of Centric Fission and Fusion in a River Buffalo (Bubalus bubalis , 2n = 50) Cow with Reduced

Fertility. Cyto. and Genom. Res., 2011; 32:26–30.

89. Pinheiro, L.E., Mikich, A.B., Bechara, G.H., Almeida. I.L. and Basrur P.K. (1990). Isochromosome Y in an infertile heifer. Genome. 33:690-695.

90. Kawakura, K., Miyake, Y., Murakami, R.K., Kondoh, S., Hirata, I.T. and Kaneda, Y. (1997). Abnormal structure of the Y chromosome detected in bovine gonadal hypoplasia (XY female) by FISH. Cytogenet Cell Genet. 76:36-38.

91. Farag, L.M., Abd El-Gawad, E.M.M., El-Naliass, E., Hassan, A.G.M. and Abd Allah, S.M. (1999). Chromosome anomalies in cattle and buffaloes in relation to reduced fertility and Uterine prolapsed with reference to early detection of chromosome anomalies in calves. J. Egypt, Ger.Soc.Zoo. 25:219-232.

92. El-Bayomi Kh M., El-Araby, I. E. and Zaglool, A. W. (2011). Cytogenetic Analysis Related to Some Infertility Problems in Cattle. Global Veterinaria. 7: 323-329.

93. Ghanem M.E., Nakao T. and Nishibori M., Deficiency of uridine monophosphate synthase (DUMPS) and X-chromosome deletion in fetal mummification in cattle. Anim. Reprod. Sci. 91:45-54.

94. Patel, R.K., Radhakrishna, U. and Khoda, V.K. (1997). Mitotic disturbance associated with variant chromosome No. 3 in river buffalo bulls (Bubalus Bubalis). Buffalo J. 3:173-178.

95. Patel, R.K. and Khoda, V.K. (1998). Presence of Variant chromosome 3 in the infertile water buffaloes (Bubalus bubalis). Veterinary Review. 13:25-27.

96. Patel, R.K. (1999). Presence of Unusual Secondary Constriction in smallest Autosome of Subfertile Murrah Buffalo Bull (Bubalus bubalis). Buffalo Newsletter.12:4-7.

97. Sangamitra K., Patel R.K., Sambasiva Rao K.R.S. and Singh K.M., Initial study on detection of fragile site on chromosomes of sub-fertile Murrah buffalo bulls, Haryana Vet., 2004; 48:71-74.

98. Prasanthi, G.S., Patel, R.K., Sambasiva, Rao K.R.S. and Singh, K.M. (2004). Detection of fragile site on chromosomes of sub-fertile cattle bulls. Intas Polivet. 5: 87-93.

99. Balakrishnan, C.R., Yadav, B.R. and Yadav, J.S. (1985). Spontaneous pericentric inversion in the Indian buffalo Bubalus bubalis. The Nucleus. 28:45–48.

100. Patel, D.J., Patel, A.J., Patel, R.K. and Parekh, P.R. (2012). Chromosomal analysis of Breeding bulls using lymphocyte culture. Bangladesh Veterinarian. 29:17-21.

101. De Lorenzi, L., Rossi, E., Genualdo, V., Gimelli, S., Lasagna, E., Perucatti, A., Iannuzzi, A. and Parma, P. (2012). Molecular characterization of Xp chromosome deletion in a fertile cow. Sex Dev. 6:298-302.

102. Oitmaa, E., Peters, M., Vaidla, K., Andreson, R., Magi, R., Slavin, G., Tonisson, N., Reimand, T., Remm, M., Schneider, M., Ounap, K., Salumets, A. and Metspalu, A. (2010). Molecular diagnosis of Down syndrome using quantitative APEX-2 microarrays. Prenat Diagn. 30:1170-1177.

103. Zhou, X., Cole, S.W., Rao, N.P., Cheng, Z., Li Y., McBride, J. and Wong, D.T. (2005). Identification of discrete chromosomal deletion by binary recursive partitioning of microarray differential expression data. J Med Genet. 42:416-9.

NEWS ITEM

Higher education system needs overhaul: Expressing concern over deteriorating quality of higher education, GD Yadav, scientist and lecturer at Institute of Chemical Technology, Mumbai, said the system needed an overhaul to avoid a mess. Explaining sustainable development, Yadav said technology should be acceptable to people and should be environment-friendly as well as economically viable. "Change is the rule of the world and the problems we face today must be solved with technology. Advanced materials need new technology, whether it is communication or genetic transformation. Unbelievable improvements have been brought about in man's life with the help of science. What seems a figment of one's imagination today will be a reality in future," he said. Yadav stressed increasing interaction between industries and academic institutions on the lines of China, Japan and the United States. He said research culture which was badly lacking in India needed to be inculcated from the school level and interest should be created among children towards science. He concluded by saying that science and technology were going to be saviours of the century.

Project to seek secrets of thinking, learning: A proposal to accelerate brain research may someday mark 2013 as the dawn of a golden era in neuroscience. In April, President Obama announced an ambitious plan to reveal the human brain's secrets. "There is this enormous mystery waiting to be unlocked," Obama said in a speech, "and the BRAIN Initiative will change that by giving scientists the tools they need to get a dynamic picture of the brain in action and better understand how we think and how we learn and how we remember."

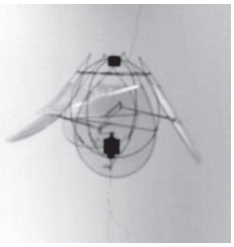
The effort will be funded by the U.S. National Institutes of Health, the Defense Advanced Research Projects Agency and the National Science Foundation, along with a host of private foundations and companies. Scientists and policy experts are still skirmishing over the focus and scope of the BRAIN Initiative, but some details are emerging. After a series of meetings with scientists around the country, a panel of neuroscientists settled on nine preliminary research priorities, ranging in scope from individual nerve cells to the entire brain.

The BRAIN Initiative will also fund scientists who develop new techniques to illuminate the coordinated behavior of large groups of nerve cells. Projects focusing on these initial nine priorities, which were approved by the NIH on September 16, are anticipated to receive $40 million of NIH funding in fiscal year 2014 And the United States–based BRAIN initiative is not alone. On January 28, the European Commission awarded 1 billion euros to the Human Brain Project, an effort by 130 research institutes to develop the most precise model of the human brain yet.

Education ministers at WISE explore innovative practices in policy making:

Education ministers from across the globe met at the World Innovation Summit for Education (WISE) 2013 in Doha, Qatar, to discuss best practices and the importance of promoting innovation in education on Tuesday. Chaired by the minister of education and higher education of Qatar, Mohammed Bin Abdul Wahed Ali Al Hammadi, the inaugural ministers session included 16 leading policymakers from territories including Japan, Nigeria, Cuba and the European Commission. The informal meeting was a transnational effort to share best practices in education from policymakers around the world who discussed a variety of ideas - from fostering innovation through curriculums,to innovative funding mechanisms.

SCIENTIFIC NEWS



Jellyfish-like flying machine takes off- Gabriel Popkin : Hummingbirds do it, bees do it. But for tiny robots, hovering has proven a challenge.Flapping-wing robots known as ornithopters can replicate insect wing motions, but these designs require complicated mechanisms to keep the machines stable. So mathematicians Leif Ristroph and Stephen Childress of New York University departed from insect mimicry. The pair designed a 2.1-gram, 10-centimeter-wide hovering machine that rises in air like a jellyfish in water.

Four teardrop-shaped flapping Mylar wings attached to a spherical shell create lift. A small motor drives a crankshaft attached by rods to each wing. Wings opposite each other flap simultaneously; the

pairs are out of phase by a quarter cycle. The result, reported January 15 in the *Journal of the Royal Society Interface*, is the first flapping-wing craft with intrinsic stability, meaning it keeps itself right-side-up without sensors or feedback controls. The machine looks innocuous enough — even cute. But potential applications of automated hovering robots are serious stuff, with surveillance, environmental monitoring and search-and-rescue topping the list.

Treatment With Beneficial Microbe Nearly Halves Crying Time- Nathan Seppa: Newborns who take drops containing a beneficial bacterium cry less than babies not given the supplement, researchers report January 13 in *JAMA Pediatrics*. The cause of excessive crying — or colic – is not well understood, but scientists suspect that the microbial mix in infants' intestines is involved. Researchers at the University of Bari Aldo Moro in Italy teamed with other scientists across Italy to randomly assign 589 newborns to get either a placebo or a probiotic supplement. The supplement contained live *Lactobacillus reuteri,* a microbe shown previously to improve intestinal function. Parents delivered the drops and kept detailed diaries of infant health for three months.

Newborns getting the microbe were less apt to develop colic symptoms. They cried for an average of 38 minutes per day; infants getting placebo cried for 71 minutes. The microbe-treated babies also spit up less often. These improvements meant fewer doctor visits and trips to emergency departments for the infants. Parents whose babies got the microbes lost only about half a day of work during the study, compared with nearly three days for parents of infants getting a placebo.

Some Animals Have Learned How To Use Plants To Heal Themselves- Sharon Oosthoek: Michael Huffman was watching a group of chimpanzees in an East African jungle when the primate researcher made a surprising observation. A mother chimp lay ill on a bed of branches in a tree as her two-year-old son climbed dangerously high. Too sick to scold or bring down her offspring, she simply ignored him. A while later, the female chimp summoned the energy to climb to the ground. She then slowly ambled over to a shrub. Huffman watched as she removed several branches. After peeling back the bark, the chimp chewed on the inner pith. Next, she sucked out the juice.

From his research at Kyoto University in Inuyama, Japan, Huffman knew a lot about chimps and their behavior. He had never seen one of the primates sample or even show interest in this shrub before. So he asked his research assistant, Mohamedi Seifu Kalunde, what it was. "*Mjonso*," said Kalunde. A member of the local Tongwe tribe, Kalunde told Huffman the bitter-tasting shrub was medicinal. He explained that members of his tribe in Tanzania used it to treat stomachaches, malarial fevers and even gut infections caused by parasites.

Suddenly, Huffman recalls of that day in 1987, "I got really excited." Huffman realized he just might have been the first university-trained scientist to watch an animal taking medicine. More specifically, he was likely the first scientist to realize the animal did so with an understanding that it was doctoring itself. Over the following years, Huffman's follow-up observations revealed other chimps using the same shrub to cure themselves of ailments. Hisdiscovery has changed the way scientists study what animals eat, says Mark Hunter. He's an ecologist at the University of Michigan in Ann Arbor.

A Shock To The Brain May Help Erase A Bad Memory-Stephen Ornes: The brain stores memories, both good and bad. But they don't always stay put. A new study suggests that receiving an electrical shock to the brain shortly after recalling a troubling event can help a person forget many of the upsetting details. The study is just the latest in a series by research teams to show that memories are plastic. That means they can be changed and sometimes even erased altogether. These findings could one day lead to new treatments for people who suffer from mental diseases like severe anxiety or post-traumatic stress disorder. Such treatments may be able to target severely disturbing memories and help patients forget them.

DNA from a bone found in Spain is the oldest yet recovered from a pre-human relative and suggests a surprising link between two types of humanlike ancestors: DNA from a 400,000-year-old leg bone is breaking records. It also raises questions about how closely ancient humanlike creatures were related to each other. The genetic material is four times older than any other recovered from a pre-human ancestor, or hominid. And scientists say the DNA links this individual, recovered from Spain, with a much later humanlike group called Denisovans. Their remains have been found only in East Asia's Siberia.The newly studied leg bone turned up nearly a decade ago. Scientists retrieved it from among the fossil remains of at least 28 individuals. All were found in a cave in Spain known as the "pit of bones." All belonged to a humanlike species that resembled people but existed long before them.

A species is a group of living things that can produce offspring that can survive and reproduce. All species — from people to boa constrictors to red maples — evolve. That means they change slowly over long periods of time. This change, or adaptation, usually occurs in response to natural variations in DNA. A long and spiral-shaped molecule, DNA is found inside almost every cell of the body. It holds the operating instructions that tell a cell what to do. Each parent passes along half of his or her DNA to any offspring. So when DNA morphs, as it often does, offspring can inherit these altered cellular instructions.

Matthias Meyer studies the DNA instructions, or genes, of ancient species at the Max Planck Institute in Leipzig, Germany. His team's new discoveries from the ancient leg bone were published Dec. 4 in *Nature*.Most scientists consider bones from the Spanish pit to be the remains of a species known as *Homo heidelbergensis.* This hominid may be an ancestor of Neandertals, an ancient species that roamed Europe. What stunned the scientists was the leg bone's genetic links to Denisovans.A finger bone and two teeth found in Siberia — far from Spain — are the only known evidence of Denisovans. And those fossils indicate Denisovans lived more than 350,000 years *after* the leg bone's owner.

Two types of DNA carry the genetic material in each individual. The form found inside a cell's nucleus contains genes inherited from both parents. A second type of DNA possesses material passed along only from a child's mother. It's found outside the nucleus, in mitochondria (MI toh KON dree ah). These are segments of a cell where energy is produced. Meyer's team focused on this mitochondrial DNA. John Hawks at the University of Wisconsin-Madison studies ancient humanlike species. The new DNA study makes a surprising connection between ancient humanlike groups, he told *Science News.* However, he said, it also raises important questions about where and how those hominids might have come together.

OPPORTUNITIES

Novozymes hiring Research Associate : Applications are invited from eligible candidates for One position of Research Associate. As a Research Associate, you will be responsible for supporting in delivery of quality results projects and good understanding of work done in the lab. This involves good coordination with team members and key stakeholders and working towards the department's growth,driving and delivering results. Candidates with M.S./ M.Sc./ M.Tech. in any branch of Life Sciences with 1-2 years of work experience can apply.

Notification for JRF post in "Cell Signaling pathway" project at IIT Bombay The Indian Institute of Technology, Bombay (IITB) is one of the Fifteen higher Institutes of Technology in the country set up with the objective of making available facilities for higher education, research and training in various fields of Science and Technology. Applications are invited from the citizens of India for filling up the following temporary position for the sponsored project undertaken in the Department of Biosciences and Bioengineering of this Institute. The position is temporary initially for a period of 1 Year and tenable only for the duration of the project. Title of the project is "Understanding protein - protein interactions of JAK-STAT pathway proteins on protein arrays" sponsored by Department of Science & Technology. One junior research fellow is available under this project. Desired profile of the aspirants should be Post Graduate (PG) Degree in Basic Sciences & NET qualified Or Graduate Degree in Professional Courses and GATE or equivalent qualification. Profile of the job is to perform molecular biology and proteomics based experiments. Prepare reports and manuscripts. Selection criteria: Assignments, written and/or interview test.Applicants having hands-on experience in molecular biology and proteomics will be preferred. Any research publication and other evidences of scientific writing will be an asset. Salary is Rs.16000.00/-p.m. Deadline for application is 27.01.14

ICGEB recruiting for RA/SRF post in HIV vaccine design research group: The International Centre for Genetic Engineering and Biotechnology provides a scientific and educational environment of the highest standard and conducts innovative research in life sciences for the benefit of developing countries. It strengthens the research capability of its Members through training and funding programmes and advisory services and represents a comprehensive approach to promoting biotechnology internationally. Applications are invited for the post of SRF/Research Associate. Desired Profile is with PhD/M.Sc Life Science /Biotechnology (PhD. Desirable but not essential). to carry out research in immunogen design for the development of vaccine(s) design for the development of vaccine(s) against HIV. Experience in determining structure of peptide based immunogen in solution particularly using NMR will be useful. Deadline for application is 30.01.14.

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MS in Pharmacy from USA

1st semester at Alliance - JNTUH in India and remaining courses & research in USA at the University of the Pacific, California, USA.

University of the Pacific, USA has entered into collaboration with JNTUH & Alliance Institute, India, for offering Masters (MS) program in Industrial Pharmaceutics. In this program students take courses in the first semester at Alliance-JNTUH and after successful completion of first semester at Alliance and fulfilling admission, TOEFL and visa requirements, students can go to USA to complete remaining courses and research at Thomas J Long School of Pharmacy and Health Sciences, University of the Pacific. Upon successful completion of the requirements, University of the Pacific will award Master's degree.

If students fail to meet University of the Pacific admission/ visa requirements, they have an option to continue their course and research work at Alliance -JNTUH or do research work at the Pacific to fulfill requirements for MS degree in India.

Admissions are based on
GPAT / PGECET / JNTUH Entrance

MS DEGREE AWARDED BY
University of the Pacific, Stockton, CA- USA

ALLIANCE INSTITUTE OF ADVANCED PHARMACEUTICAL AND HEALTH SCIENCES

#604A, Aditya Trade Centre, Ameerpet, Hyderabad – 500 038, India
Phone: 040-66663326 / 23730072, Website: www.allianceinstitute.org

About Alliance: Alliance, located conveniently in the heart of Hyderabad, trains industry-ready graduates by bridging education with industry needs in pharmaceutical sciences. Alliance's visionary management built state of the art facilities and laboratories to provide quality education meeting national and international standards.

Collaboration with JNTUH, India: Alliance is having collaboration with **Jawaharlal Nehru Technological University, Hyderabad (JNTUH)**, which is a premier institution with academic and research–oriented programs, offered through the constituent and affiliated colleges. Alliance's syllabi, academic regulations and course structure are **approved by the JNTUH. JNTUH awards the degrees after fulfilling the degree requirements.**

Collaboration with University of the Pacific, USA: University of the Pacific, ranks in the top 100 among the 3000 national universities in the United States. Alliance has entered into research collaboration with Thomas J Long School of Pharmacy and Health Sciences, University of the Pacific.

Alliance students have an option to do research work at the University of the Pacific to fulfill requirements for MS degree in India. Pacific faculty teaches Alliance students via live online classes. Pacific is also interested to offer admissions to Alliance students based on their performance at Alliance.

Programs offered :

* MS in Industrial Pharmaceutics
* MS in Pharmaceutical Analysis & Quality Control
* MS in Drug Development & Regulatory Affairs

For admissions, application forms and additional information visit online at www.jntuh.ac.in/alliance or www.allianceinstitute.org.

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