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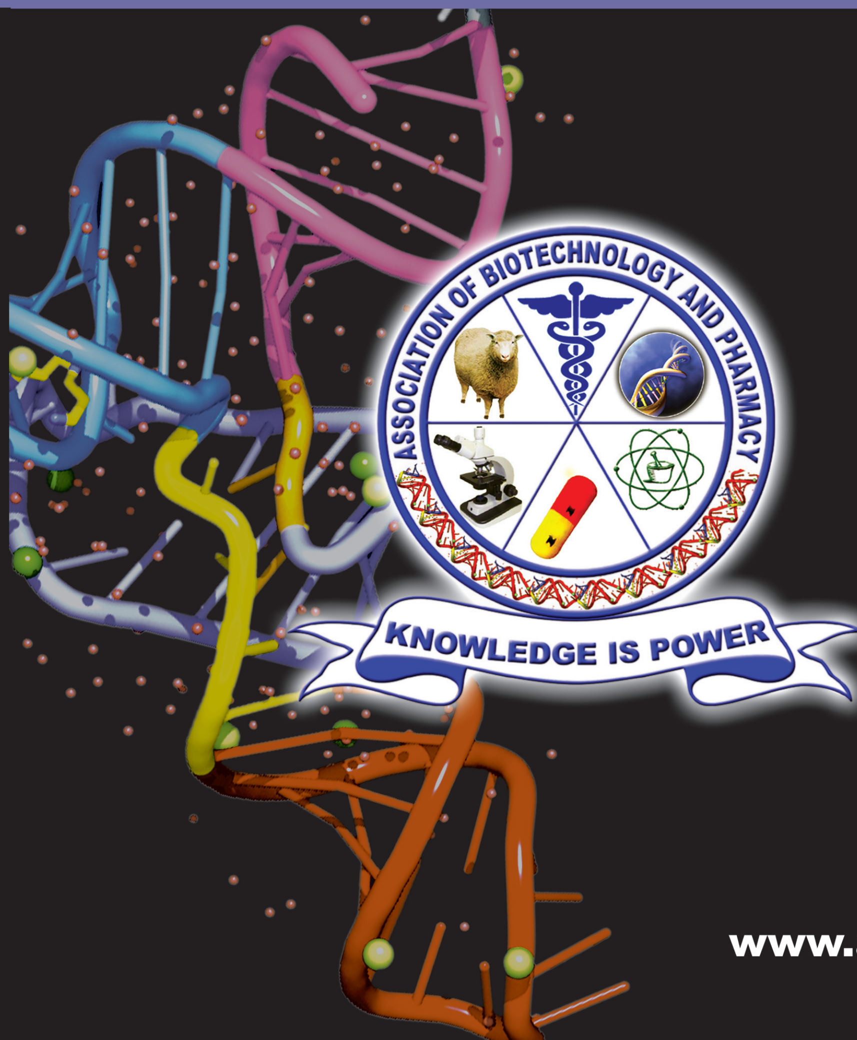

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Mahavadi, S., Rao, R.S.S.K. and Murthy, K.S. (2007). Cross-regulation of VAPC2 receptor internalization by m2 receptors via c-Src-mediated phosphorylation of GRK2. Regulatory Peptides, 139: 109-114.

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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Selection of Salt Tolerant Cowpea Genotypes Based on Salt Tolerant Indices of Morpho-biochemical Traits

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Abstract

Soil salinity is one of the abiotic factors affecting crop production worldwide. Introducing salt tolerant genotypes is considered as an economical strategy for increasing the production of agricultural crops in salt affected soils. In this study, morphological and biochemical changes of twenty three cowpea genotypes from India were evaluated for salt tolerance using seven days old seedlings treated with 0 and 75mM sodium chloride solutions. Chlorophyll, sugar, proline, soluble protein, sodium (Na^+), potassium (K^+), K^+/Na^+ ratio, shoot length, root length and fresh weight were determined and all observations were converted to salt tolerance indices (STI). Chlorophyll content, K^+/Na^+ ratio, and growth parameters decreased on exposure to salt stress; whereas sugar, proline and soluble protein contents increased. To improve the selection efficiency of salt tolerant genotypes, STI values of all parameters were subjected to hierarchical clustering and the 23 genotypes were grouped into three clusters. Nine salt tolerant genotypes (KBC2, IVT-VCP-09-013, VBN1, VBN2, CO(CP)7, VCP-09-001, DC15, PGCP5 and VCP-09-030) were identified by hierarchical clustering. Correlation studies showed that soluble protein content and K^+/Na^+ ratio positively correlated with all the growth parameters indicating their major contribution towards salt tolerance.

Keywords: Cowpea, salt tolerance, proline, K^+/Na^+ ratio, cluster analysis

Introduction

Cowpea (*Vigna unguiculata* (L) Walp), is an important pulse crop in semi-arid tropics covering Asia, Africa, southern Europe and Central and South America. It is grown throughout India for its long, green vegetable pods, grains, and foliage for fodder. The grain contains about 25% protein and 64% carbohydrate (1) and serves as a source of cheap protein for both rural and urban consumers. In India, it is grown in an area of about 3.9 million hectares with productivity of 567 kg per hectare (2). But, the average cowpea yield is very much less than the estimated potential yield. The major reasons for low yield are climatic, biotic, abiotic, and technological problems. Soil salinity is one of the most severe abiotic stresses affecting production of legumes worldwide (3). Cowpea is grouped as a moderately salt tolerant crop (4).

Salinity is one of the major hazards, usually confined to arid and semi-arid regions of the world (5). Nearly 20% of the world's cultivated area and nearly half of the world's irrigated lands are affected by salinity (6). Soil salinity is a serious threat worldwide for sustainable agricultural production (7, 8). In the Indian context, salt affected soils occupy about 6.73 million hectares affecting production and productivity across a number of states (9). In addition to this, use of poor quality irrigation water also causes salt stress to the agricultural crops (10). Though chemical amelioration can reduce productivity losses, the availability of soil amelioration chemicals is often limiting in interior villages and

also chemical amelioration is costly and thus out of reach for small and marginal farmers. Hence, introducing salt tolerant genotypes is considered as an eco-friendly, economical and socially acceptable feasible strategy for increasing the production in salt affected soils (11).

Salinity affects seed germination, plant growth, nutrient uptake, and metabolism owing to osmotic inhibition of water availability, ion imbalance, toxic effect of salt ions, and their effects on cellular gene expression machinery. Different plant species have developed different mechanisms to cope up with salinity stress effects. It is accepted that non-toxic compatible organic solutes accumulate in the cytoplasmic compartment of cells and inorganic ions toxic to metabolic process are restricted to the vacuoles. The progress in developing salt tolerant crop varieties has been very slow due to our incomplete knowledge of the mechanism of salt damage and the complex nature of salt tolerance. The differential response of plants to salt stress at different growth stages has added further problems in this direction (12, 13).

To improve the reliability and selection efficiency for salt tolerance, it is necessary to identify the salt induced characteristic changes in multiple traits among different genotypes. This study compared seedling growth parameters and biochemical traits, as well as their relationship with salt tolerance. The STI value was considered as the indicator for salt tolerance. Through comparing genetics and correlation of STI, we have obtained critical information about salt tolerance in different cowpea genotypes. Findings of this study will be useful for screening salt tolerant cowpea genotypes from germplasm and also provide information for breeding tolerant cultivars.

Materials and Methods

***Seed materials*:** Seeds of twenty three cowpea genotypes obtained from different places in India were used in this study. Twelve genotypes (CPD121, PGCP6, KBC5, CoVu702, PGCP5, GC3, NBC5, GC0817, PGCP12, DC15,GC521 and KBC2) were obtained from Central Arid Zone Research Institute, Jodhpur; three genotypes ACM002, CP16 and CO(CP)7 from Agricultural College and Research Institute, Madurai. The remaining eight genotypes (VBN1, VBN2, VCP-09-001, IVT-VCP-09-013, VCP-09-016, VCP-09-030, VCP-09-019 and VCP-09-035) were from National Pulses Research Centre, Vamban.

***Selection of salt concentration*:** Five cowpea genotypes (COCP7, CP16, CPD121, DC15, GC0817) were chosen on random basis and used for selection of salt concentration that will be ideal for carrying out further screening of salt tolerant genotypes. Germination was conducted by the standard roll towel method (14). Seeds were first surface sterilized in 70% ethanol for 2 minutes and rinsed thoroughly with sterile distilled water. Fifteen sterilized seeds were placed on a pre-soaked germination paper placed over a polythene sheet. The seeds were held in position by another pre-soaked germination paper strip and gently pressed. This was rolled separately for each treatment and kept vertically inside troughs containing 0 (distilled water), 50, 75, 100 and 125 mM NaCl solutions in germination room maintained at 28 ± 1°C and 80% of relative humidity. A completely randomized design with five cowpea genotypes subjected to five treatments and five replications was adopted. Shoot length and root length of five seedlings from each replication were measured on the 7th day after sowing. Based on this experiment, 75 mM NaCl concentration was selected for carrying out further experiments.

***Screening for salt tolerant genotypes*:** Seeds of twenty three different cowpea genotypes were first surface sterilized and allowed to germinate by roll towel method as described above and subjected to 0 mM (distilled water) and 75 mM NaCl solutions. The temperature was maintained at 28 ± 1°C with relative humidity of 80%. A completely randomized design with 23 cowpea genotypes subjected to two treatments and three replications was adopted. Seedlings were harvested on the 7th day after sowing and morphological and biochemical observations were recorded.

***Morphological parameters*:** Morphological parameters such as shoot length, root length and fresh weight of the seedlings were measured and the mean value worked out.

***Mineral Constituents*:** Sodium (Na^+) and Potassium (K^+) contents were analysed in primary leaf alone to ensure measurements were focussed on the effects of these ions on the primary photosynthetic tissues. The leaves were harvested, dried at 65°C and the dry weights were determined. The dried plant samples were powdered. About 0.5g of the sample was wet digested with 10 ml of nitric acid: perchloric acid (4:1) mixture. After completion of digestion, the solution was transferred to a 25 ml volumetric flask and made up to the mark with deionized water. Na^+ and K^+ contents were determined by flame photometry (15). From this, K^+/Na^+ ratio was calculated.

Biochemical analysis: All biochemical analysis was conducted in fresh primary leaf samples. Photosynthetic pigments such as chlorophyll a, b and total chlorophyll were estimated by spectrophotometric method (16). About 0.2 g of leaf sample was extracted with 80% acetone and made up to 25 ml and read the absorbance at 645 and 663 nm in a spectrophotometer. Chlorophyll (Chl) contents were determined using the following equations where A_{645} and A_{663} are the absorbance at 645 and 663 nm respectively.

$$\text{Chl a (mg L}^{-1}) = (12.7 \times A_{663}) - (2.69 \times A_{645})$$
$$\text{Chl b (mg L}^{-1}) = (22.9 \times A_{645}) - (4.68 \times A_{663})$$
$$\text{Total Chl (mg L}^{-1}) = (20.2 \times A_{645}) + (8.02 \times A_{663})$$

Total soluble sugar was estimated by anthrone method (17) using glucose as standard. Weighed 0.2 g of the fresh leaf sample and extracted with 80% ethanol and the supernatant evaporated to dryness in a hot water bath at 80°C. The residue was dissolved and made up to 25 ml with distilled water. To 1.0 ml of the extract, 4 ml anthrone reagent (0.2% anthrone in 95% H_2SO_4) was added and cooled in ice, then kept in boiling water bath for 8 minutes and absorbance measured at 630 nm.

Proline was estimated by acid ninhydrin method using proline as standard (18). Fresh leaves (0.5 g) were extracted with 10 ml 3% sulphosalicyclic acid and filtered. To 2 ml of the filtrate, 2 ml of acid ninhydrin reagent (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml orthophosphoric acid) and 2 ml glacial acetic acid were added and kept in boiling water bath for 1 hour and cooled in ice bath. Toluene (4 ml) was added and mixed vigorously and the upper coloured toluene layer was separated and absorbance measured at 520 nm against toluene as blank using spectrophotometer.

Total soluble protein content was estimated by Lowry's method using bovine serum albumin as standard (19). Leaf sample (0.2 g) was homogenised with 0.1 M phosphate buffer (pH 7.0) and centrifuged. To 0.1 ml supernatant, 2.4 ml distilled water and 5 ml of alkaline copper reagent were added. Incubated at 37°C for 10 minutes and added 0.5 ml of Folin Ciocalteau reagent and again incubated at 37°C for 20 minutes. Absorbance was measured against reagent blank at 620nm.

***Data processing and statistical analysis*:** All observed data were converted to salt tolerance indices (STI). A salt tolerance index is defined as the observation at salinity divided by the average of the controls (20, 21). The data were analysed for significance by ANOVA using SPSS v.20 software. The STI values of the different traits were subjected to correlation analysis (Pearson) using SPSS v.20 software. After conversion of STI values into non-dimensional data matrices using the subordination method in fuzzy mathematics (22), hierarchical cluster analysis based on squared Euclidian distance and Ward's linkage was performed using SPSS v.20 software.

Results

Effect of different salt concentrations on growth: The preliminary study showed that increasing the salt concentration reduced both shoot and root length in cowpea seedlings as shown in figure-1. At 75 mM NaCl concentration,

CPD121 showed 50.83% reduction in shoot length as given in table-1. At 100 mM NaCl concentration, all genotypes except GC0857 showed more than 50% reduction in shoot length. All genotypes showed greater than 64.72% reduction in shoot length when exposed to 125 mM NaCl.

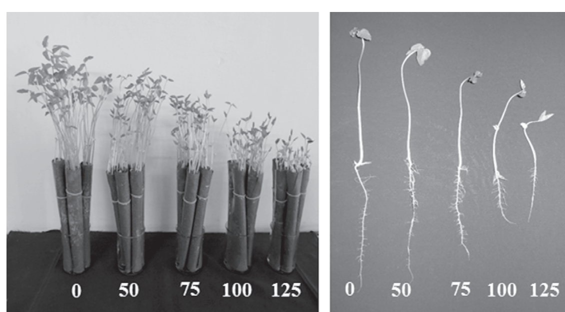

Fig. 1. Effect of salt stress on shoot and root growth. Reduction of shoot and root length observed in DC 15 cowpea seedlings subjected to 0, 50, 75, 100, and 125 mM NaCl solutions.

NOTE: Each value for shoot length represents five replications ± Standard deviation

Root length also decreased with increasing NaCl concentration (table-2). At 75 mM NaCl concentration, reduction in root length ranged between 18.31 and 48.94%. Root length reduced drastically above 100 mM NaCl concentration. All genotypes showed more than 56.47% reduction in root length when subjected to 100 mM NaCl concentration.

Salt tolerance indices for different traits: Biochemical and growth parameters were determined for 23 cowpea genotypes and the STI values were calculated. The relative STI for all 12 measured parameters varied among genotypes (table-3). Seedlings of VBN2 and GC0817 had significantly higher STI value for shoot length. VBN2 showed highest STI for root length, but was on par with that of VCP-09-035, PGCP5, DC15, VBN1 and VCP-09-030. Lowest STI for shoot length was observed in PGCP12. Genotypes CP16, PGCP12 and GC521 showed statistically lower STI for root length. Seedlings of PGCP6, PGCP5, NBC5, KBC2, VCP-09-030, VCP-09-035, VBN2, VCP-09-019, IVT-VCP-09-013, GC521 and VBN1 showed minimal reduction in fresh weight and STI values were high ranging between 0.94 and 0.82; whereas, fresh weight reduction was higher in CPD121, PGCP12 and ACM02 with lower STI values ranging between 0.59 and 0.69.

Salt stress caused significant reduction in chlorophyll contents which is reflected in the STI values (table-3); especially chlorophyll a decreased more obviously than chlorophyll b. KBC2 and CO(CP)7 had higher chlorophyll a and total chlorophyll contents; PGCP5 and VCP 09 030 had higher chlorophyll b contents. STI values for total soluble sugar varied between 1.69 and 1.03 among genotypes. However, the proline indices showed wide variation between genotypes and ranged between 2.46 and 1.06. Proline indices for VBN1 and DC15 were two

Table 1. Effect of salt stress on shoot length of cowpea genotypes

	Shoot length (cm)					Reduction (%)			
Genotype	Salinity levels (mM NaCl)								
	0	50	75	100	125	50	75	100	125
CO(CP)7	23.56±0.94	18.14±0.67	16.34±0.69	9.72±0.31	6.24±0.29	23.00	30.64	58.74	73.51
CP16	23.22±0.95	20.10±0.37	11.78±0.59	9.02±0.59	3.94±0.27	13.44	49.27	61.16	83.03
CPD121	20.50±0.81	14.14±0.48	10.08±0.48	7.14±0.58	4.86±0.13	31.02	50.83	65.17	76.29
DC15	24.24±0.77	18.72±0.67	16.64±0.59	10.88±0.78	6.88±0.30	22.77	31.35	55.12	71.62
GC0817	21.12±0.47	18.98±0.32	17.68±0.78	11.38±0.41	7.86±0.57	13.86	19.57	48.92	64.72

times higher than NBC5 and PGCP12. Total soluble protein indices also showed wide variation with values between 1.29 and 2.74. VBN1 had two times higher protein index than ACM02 and CP16.

Salinity affected Na^+ and K^+ contents of cowpea genotypes. Increase in Na^+ content was observed and STI values ranged between 1.59 and 3.45. CP16 accumulated more Na^+ and PGCP5 showed least accumulation. High K^+ content and K^+/Na^+ ratio was observed in CO(CP)7, DC15, VCP-09-030 and VCP-09-001 genotypes. PGCP5 and VCP-09-035 also showed higher K^+/Na^+ ratio. Lower values for K^+/Na^+ ratio was observed in CP16, CPD121 and PGCP12 genotypes. K^+ content decreased in three genotypes PGCP6, PGCP5 and PGCP12 under salt stress, whereas, increased in other genotypes. STI values for K^+ ranged between 0.92 and 1.61.

Cluster analysis

Hierarchical clustering using squared Euclidian distance and Ward's linkage formed three clusters (I, II and III) to characterize salt tolerance of cowpea genotypes at seedling stage (figure-2). Cluster I represented the salt sensitive genotypes (CPD121, ACM002, CP16 and PGCP12) which showed lower STI values for the different traits estimated. Moderately salt tolerant genotypes were grouped in cluster III and tolerant genotypes in II (KBC2, IVT-VCP-09-013, VBN1, VBN2, CO (CP)7, VCP-09-001, DC15, PGCP5 and VCP-09-030). Cluster means of STI values for all the traits were calculated and were represented graphically (figure-3). Generally tolerant genotypes in cluster II showed higher cluster mean values of STI for the morphological and biochemical parameters. It was observed that cluster means for proline and total soluble protein contents were comparatively high in

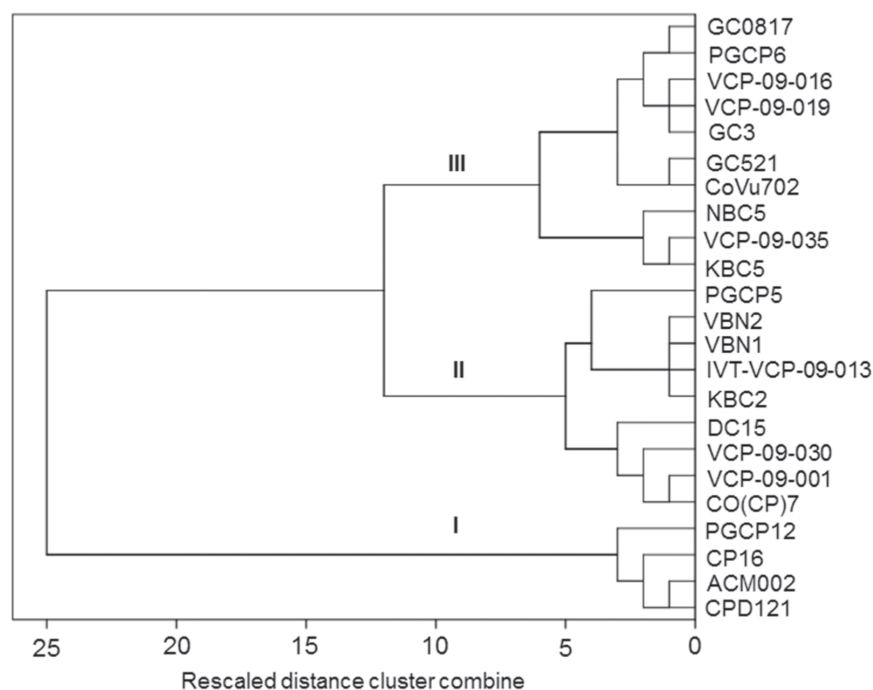

Fig. 2. Dendrogram of cowpea genotypes clustered for salt tolerance using Ward linkage. The 23 cowpea genotypes were grouped into three clusters. Cluster I represents the salt sensitive genotypes; cluster II, salt tolerant and cluster III, moderately salt tolerant.

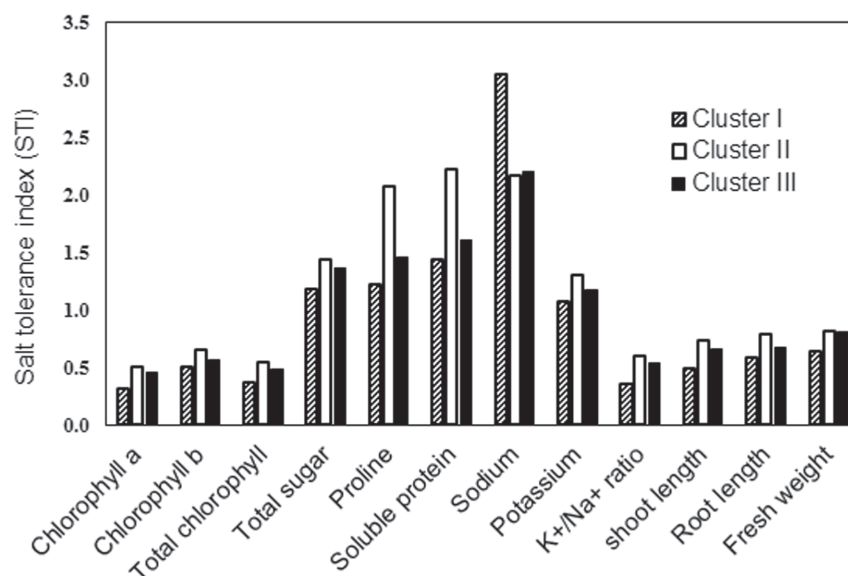

Fig. 3. Cluster means for the biochemical and growth traits. Proline and soluble protein contents are relatively higher in the salt tolerant cluster II, whereas salt sensitive cluster I has higher accumulation of sodium ions.

Table 2. Effect of salt stress on root length of cowpea genotypes

Genotype	Root length (cm)					Reduction (%)			
	Salinity levels (mM NaCl)								
	0	50	75	100	125	50	75	100	125
CO(CP)7	22.06±1.15	19.82±0.31	14.68±0.34	9.50±0.31	6.78±0.48	10.15	33.45	56.94	69.26
CP16	17.90±1.10	15.78±0.22	9.14±0.63	7.06±0.27	4.82±0.31	11.84	48.94	60.56	73.07
CPD121	22.08±0.36	16.92±0.71	15.06±0.84	5.90±0.46	1.80±0.42	23.37	31.79	73.28	91.85
DC15	16.82±0.57	15.10±0.27	13.74±0.23	6.90±0.51	6.26±0.15	10.23	18.31	58.98	62.78
GC0817	23.48±0.95	20.04±0.54	14.68±0.70	10.22±0.60	6.84±0.69	14.65	37.48	56.47	70.87

NOTE.- Each value for root length represents five replications ± Standard deviation

cluster II, whereas Na^+ accumulation was higher in cluster I.

Discussion

Reduction in growth: The present study showed that salinity reduced shoot length, root length and fresh weight of cowpea seedlings; salt-sensitive genotypes CPD121, CP16, PGCP12 and GC521 showed comparatively more reduction in growth parameters than tolerant genotypes. Previous studies also reported growth reduction during salt stress (23). This study showed that Na^+ content was negatively correlated with shoot and root length as well as fresh weight. Generally, growth reduction occurs due to salt-induced osmotic stress (24), hardening of the cell wall (25) and a decrease in conductance of the plasma membrane (26, 27). Growth reduction might be partly due to stomatal closure which limits CO_2 assimilation and

Table 3. Salt tolerance indices for individual traits in 23 cowpea genotypes

Genotype	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}
CPD121	0.28	0.46	0.31	1.23	1.30	1.54	3.03	1.08	0.36	0.50	0.67	0.59
PGCP6	0.52	0.60	0.54	1.40	1.31	1.78	2.06	0.94	0.46	0.70	0.71	0.94
ACM002	0.28	0.43	0.31	1.19	1.18	1.29	3.22	1.31	0.41	0.57	0.69	0.67
KBC5	0.36	0.48	0.39	1.20	1.65	1.48	2.07	1.20	0.58	0.69	0.75	0.78
CoVu702	0.46	0.66	0.51	1.21	1.84	1.49	2.60	1.10	0.42	0.70	0.70	0.74
PGCP5	0.34	0.80	0.44	1.59	1.95	1.99	1.59	0.99	0.62	0.74	0.85	0.91
GC3	0.50	0.62	0.54	1.47	1.29	1.60	2.15	1.26	0.59	0.68	0.64	0.75
NBC5	0.38	0.51	0.42	1.03	1.10	1.74	2.05	1.17	0.57	0.61	0.60	0.88
GC0817	0.52	0.65	0.55	1.69	1.31	1.86	2.28	1.04	0.45	0.84	0.66	0.78
CP16	0.29	0.59	0.37	1.31	1.37	1.38	3.45	1.00	0.29	0.50	0.49	0.69
PGCP12	0.43	0.57	0.48	1.03	1.06	1.54	2.47	0.92	0.37	0.40	0.50	0.63
VBN1	0.55	0.62	0.57	1.44	2.46	2.74	1.89	1.07	0.57	0.71	0.84	0.82
VBN2	0.52	0.66	0.56	1.31	2.26	2.20	2.34	1.09	0.47	0.86	0.88	0.84
DC15	0.44	0.52	0.46	1.28	2.46	1.66	2.44	1.56	0.64	0.71	0.85	0.73
GC521	0.52	0.67	0.55	1.24	1.64	1.45	2.44	1.41	0.58	0.55	0.56	0.83
KBC2	0.63	0.67	0.64	1.42	1.99	2.40	2.18	1.16	0.53	0.73	0.73	0.87
CO(CP)7	0.58	0.67	0.60	1.51	1.81	2.58	2.28	1.61	0.70	0.71	0.68	0.73
VCP-09-001	0.54	0.55	0.55	1.62	1.99	2.12	2.30	1.45	0.63	0.65	0.69	0.79
IVT-VCP -09-013	0.54	0.72	0.58	1.27	2.06	2.33	2.32	1.32	0.57	0.68	0.73	0.83
VCP-09-016	0.50	0.54	0.51	1.63	1.79	1.52	2.00	1.03	0.52	0.55	0.66	0.77
VCP-09-030	0.44	0.75	0.52	1.55	1.72	1.96	2.17	1.50	0.69	0.79	0.83	0.85
VCP-09-019	0.42	0.55	0.45	1.58	1.48	1.68	2.22	1.25	0.56	0.64	0.68	0.84
VCP-09-035	0.41	0.46	0.42	1.27	1.27	1.53	2.15	1.40	0.65	0.70	0.85	0.85

NOTE.- X_1- Chlorophyll a, X_2- Chlorophyll b, X_3- Total chlorophyll, X_4- Total soluble sugar, X_5-Proline, X_6-Soluble protein, X_7-Sodium, X_8-Potassium, X_9-K+/Na+ ratio, X_{10}-Shoot length, X_{11}-Root length, X_{12}-Fresh weight.

reduced photosynthetic rate which in turn limited the supply of carbohydrate needed for growth (28, 29). Our study showed the reduction of chlorophyll pigments under salt stress which also contributed to less photosynthetic rate and hence growth reduction.

Contribution of biochemical traits to salt tolerance: Salt stress cause altered metabolism which leads to accumulation or depletion or changes in biochemical constituents. It was observed that salt tolerant genotypes had relatively higher chlorophyll a content (KBC2 and CO(CP)7) and chlorophyll b contents (PGCP5 and VCP-09-030) than salt sensitive genotypes. Correlation study showed positive correlation of chlorophyll a, b and total chlorophyll contents with shoot length and fresh weight. This showed that higher the chlorophyll content during salt stress, more will be its salt tolerant capacity. Decrease in chlorophyll content under salt stress had already been reported in different crops (30).

Accumulation of soluble sugars was observed in our study in all genotypes with varied concentration. Soluble sugars play a lead role in osmoprotection, carbon storage and radical scavenging indicating its partial role in salt

Table 4. Correlation of STI for 12 traits in cowpea genotypes

Trait	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}
X_1	1											
X_2	.468**	1										
X_3	.973**	.654**	1									
X_4	.360**	.364**	.386**	1								
X_5	.443**	.374**	.467**	.252*	1							
X_6	.626**	.493**	.661**	.334**	.617**	1						
X_7	-.450**	-.354**	-.474**	-.333**	-.294*	-.438**	1					
X_8	.197	-.033	.146	.080	.247*	.143	.012	1				
X_9	.375**	.226	.368**	.305*	.367**	.389**	-.657**	.719**	1			
X_{10}	.402**	.407**	.442**	.401**	.476**	.497**	-.463**	.195	.444**	1		
X_{11}	.110	.146	.118	.226	.571**	.390**	-.407**	.240*	.460**	.670**	1	
X_{12}	.343**	.368**	.384**	.209	.215	.303*	-.595**	.002	.418**	.529**	.373**	1

NOTE.- X_1- Chlorophyll a, X_2- Chlorophyll b, X_3- Total chlorophyll, X_4- Total soluble sugar, X_5-Proline, X_6-Soluble protein, X_7-Sodium, X_8-Potassium, X_9-K+/Na+ ratio, X_{10}-Shoot length, X_{11}-Root length, X_{12}-Fresh weight. Correlation coefficient significant at $^{**}P < 0.01$ and $^{*}P < 0.05$

tolerance (12). Sugar content positively correlated with chlorophyll content indicating the role of photosynthesis in sugar accumulation.

The accumulation of nitrogen-containing compatible solutes including proline is known to function in osmotic adjustment, protection of cellular macromolecules from damage by salts, storage of nitrogen and scavenging of free radicals. The present study showed accumulation of proline in cowpea seedlings under salt stress. Tolerant genotypes were able to accumulate twice the amount of proline when subjected to salt stress. Proline accumulation was previously observed under salinity in many plants including maize (31), sorghum (32), green gram (33) and mulberry (34). Proline content showed positive correlation with both shoot and root length, but not with fresh weight. Thus, proline content showed more contribution towards salt tolerance than sugar content. Also the high cluster mean value of proline for the salt tolerant cluster confirms its contribution to salt tolerance.

Salt stressed cowpea seedlings expressed higher total soluble protein levels with variation among genotypes. Proteins that accumulate in plants grown under saline conditions may provide a storage form of nitrogen that is re-utilized when stress is over and may play a role in osmotic adjustment (12). Proteins may be synthesized by *denovo* in response to salt stress or may be present constitutively at low concentration and increase when plants are exposed to salt stress. Our study showed that salt tolerant genotypes had higher protein content than salt sensitive genotypes. The cluster mean value for salt tolerant cluster was markedly higher for protein. Also, protein content positively correlated with all growth parameters andour study proved its major contribution in overcoming salt stress.

Changes in mineral contents

In most of the genotypes Na^+ content increased to more than two times, but was much higher in sensitive genotypes. This study proves the deleterious effect of sodium accumulation. We observed decrease in K^+ content in 3

genotypes, whereas increased in other genotypes. It was observed that K^+/Na^+ ratio decreased under salt stress. Increase in K^+ content was observed in leaves of *Butea monosperma* seedlings exposed to salt stress (35). Several studies reported that salt tolerant plants showed higher K^+/Na^+ ratio compared to salt sensitive plants (36, 37). Our study also showed that some tolerant genotypes such as DC15, CO(CP)7 and VCP-09-030 and a moderately tolerant genotype VCP-09-035 had higher K^+/Na^+ ratio compared to sensitive genotypes under salt stress. Positive correlation of K^+/Na^+ ratio with all growth and biochemical parameters showed that K^+/Na^+ ratio had a major contribution towards salt tolerance.

Conclusion

Salt tolerance is a complex phenomenon contributed by multiple biological parameters in plants. The current study on salt tolerance and associated traits of cowpea seedlings suggested that soluble protein content and K^+/Na^+ ratio contributed more towards salt tolerance since these traits positively correlated with the growth parameters. Moreover this study enabled us to identify nine salt tolerant cowpea genotypes *viz.* KBC2, IVT-VCP-09-013, VBN1, VBN2, CO (CP) 7, VCP-09-001, DC15, PGCP5 and VCP-09-030. Detailed studies on gene expression patterns associated with the above traits will be useful to strengthen our knowledge on biochemical basics of the salt tolerance in cowpea.

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Optimization of real time PCR for checking the activity of siRNA in Dengue Serotypes

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Abstract

Dengue, the most prevalent infectious disease among the arbovirus community is emerging as a first order health complication, especially, in the tropics and in subtropical regions. It is untreatable, since, there is no advanced diagnostic method with which tendency of the infection can be traced with great precision in its acute phase. In our previous studies we showed that, siRNA targeting the 5NTR among all the 4 serotypes of dengue successfully inhibited the replication of respective serotypes to the greatest extent as confirmed by the real-time RT-PCR. In the present study, we monitored the relative expression of dengue genomic RNA in treated (rAdsh-5b) and non treated cells and control (rAdsh-N) to reveal the efficiency of the real-time RT-PCR in detecting the viral load in the presence of antiviral agent (rAdsh-5b) to discriminate the disease severity in the presence and absence of antiviral therapeutics. The cDNA for all the samples were prepared and performed real-time RT-PCR with the aid of SYBR green 1 and the post PCR amplicons were analyzed with melting curve analysis to check the specificity and efficiency of the process. The results showed that, the relevant viral RNA expression was found to be less in rAdsh-5b treated sample than the non treated sample as marked by the Ct-values and the melting curve peaks. This indicates that real-time RT-PCR can serve as a gold standard for diagnosing the dengue infection in the acute phase and in distinguishing the relative viral content in the sample treated with antiviral agent.

Key Words: Dengue, siRNA, real-time RT-PCR, rAdsh-5b, rAdsh-N, Ct-values, cDNA.

Introduction:

Dengue, being a fatal infectious disease is spreading around the globe very rapidly due to the uncontrolled migration from the dengue endemic places and lack of mosquito controlling methods. The dengue virus incidences are being increased tremendously in the recent years, expanding severity with the wide array of vector(s). The contagious viral activity is attributed to the fact that, the clinical presentation of the infections is asymptomatic and sometimes undifferentiated. Hence, clinical manifestation is required to halt the progression of the disease. The dengue viral infection can be diagnosed either by direct method or by indirect method which include genome detection, antigen detection, virus isolation and serological methods (aided in IgM and IgG detection) respectively (1-6). Since the virus lasts in the infected tissue for 0-14 days bearing undifferentiated symptoms, the reliability of each method lies mainly on the type of the specimens being used and the stage of the infection.

In the recent years, the field of molecular diagnosis based on the reverse transcription PCR has become the standard tool due to its

sensitivity, reliability and specificity in the detection of acute phase infections of life threatening diseases, which in turn replaced the conventional methods. Since the emergence of RT-PCR, many research laboratories have reported various protocols for the detection of dengue virus in wide range of samples (7-9). In fact, two step nested RT-PCR method discribed by Lanciotti *et al.* and Laue *et al.* (10, 11) and single step multiplex RT-PCR by Henchal et al. (8) were the reference protocols. The conventional PCR studies described so far confirmed the persistence of virus in the disease state by detecting the viral load at a single point of time. Since viral load is a direct marker of the disease severity there is an urge in monitoring disease progress in the real-time by detecting the viral persistence from the acute phase. However, it has been postulated that monitoring the viral replication over time could be an efficient marker of a progressive viral disease rather than determining the absolute viral load at a single point of time (12, 13). This statement limited the conventional PCR methods which are solely depends on the end point analysis. With the development of fluorescent chemistries such as SYBR green, Taqman probes, linear and hairpin oligoprobes and self-fluorescing amplicons (12, 13) in the recent days, many investigators have developed the real time RT-PCR methods for the rapid diagnosis of dengue in clinical samples by reducing the time and cost in real time compare to the previously described RT-PCR and other conventional methods (14-17). There are many real-time RT-PCR methods utilizing different combinations of primers, probes and fluorescent chemistries in detecting, serotyping, and quantification of dengue virus in various clinical samples have been described (18-22). However, SYBR green and Taqman probes were most widely used detection dyes among fluorescent chemistries. The real time RT-PCR protocols reported so far in the present study are based on the consensus sequences of the dengue genome such as NS5b coding region, Capsid region, 3^1 Non-coding and 5^1Non-coding sequences with the ease of either SYBR green1 or Taqman probes as a marker dye (23, 24). In the recent decades, the real time PCR methods expanded their level of detection not only by addressing different clinical specimens, but also by targeting different regions of the dengue viral genome.

Though, Lanciotti *et al.* (10) developed nested RT-PCR in which the primers randomly target various regions of the dengue genome to detect and differentiate the dengue serotypes in the clinical samples (21), the experiments concluded with the false negative results due to the cross contamination and multiple steps involved. To avoid these false results, recent methods have been designed in selecting the primers from gene bank targeting only the conserved regions of the genome and profound use of these modified primers against the mutating viral strains (25). In the present study, we selected the consensus primers primer D1 and Primer D2 common to conserved regions of all serotypes of the dengue genome and The severity of virus infection in the presence and absence of the antiviral molecule rAdsh5b which is designed to target the 5^1UTR conserved sequences of the dengue is monitored based on the SYBR Green one real time RT-PCR and specificity of the method is confirmed using melting curve analysis. In an ultimate sense, we aimed at differentiating viremic spike in the infected cell culture and the suppressed state of the same viral entity in response to the antiviral molecule rAdSh5b.

Materials and Methods

***Viruses, Plasmid and Cell lines selection*:** The four Dengue virus serotypes are DENV-1, DENV-2, DENV-3 and DENV-4 (U88535, Nauru Island, AF038403-New Guinea C, M93130-H87, M14931-Dominica) and monkey kidney Vero cell line from ATCC, Virginia, USA for virus infection and rAdsh5b plasmid targeting conserved sequences in the 5^1-UTR region of the above mentioned virus strain's genome (26).

Primer selection based on the 5^1-UTR sequence alignment : The 5^1-UTR of the above DENV strains were aligned using ClustalW

sequence alignment software and the conserved set of nucleotides among all DENV were selected. The consensus primers D1 and D2 were selected based on the criteria that, the primers should have maximum homology with the above selected sequences and must avoid pairing with non-similar sequences to reduce the false results as described in the earlier studies (22).

***Virus infection and RNA extraction*:** The Vero cells were seeded in 12-well plate 48hr before the infection with a seed density of $0.1x10^6$/ml. When the cells attained desirable confluency, spent media was removed and 250 µl of rAdsh-N contained in 2% ΔFCS+1X DMEM was added from B1 to B4 columns, rAdsh5b from C1 to C4 columns and returned to incubator. After 2hrs of incubation, 750µl of 5 ΔFCS+1X DMEM was added to make the final volume to1000µl and placed in the incubator to finish day-1. After 24hrs of the rAdsh infection, DENV infections were setup from column A to C as follows. The medium was removed from each well and 250 µl of virus diluent DENV-1 (A1 to C1), DENV-2 (A2 to C2), DENV-3 (A3 to C3) and DENV-4 (A4 to C4) was added and the plates were returned to the incubator. After 2hs, 750 µl of 5% ΔFCS+1X DMEM was added to make the final volume to 1000 µl and replaced in the incubator to complete day-2. After every 24hrs, designated as day 3, day 4, day 5, day 6, day 7, day 8, day 9 100 µl of cell culture supernatant were collected and stored at -80^{0}C until use. The RNA from each day sample is harvested using Trizol reagent (Invitrogen). In brief, cells were washed with 1ml of 1XPBS and lysed the cells by adding trizol reagent and mixed vigorously by adding chloroform for 15 seconds and then centrifuged. RNA from the aqueous phase was precipitated by adding isopropanol and centrifuged at high speed. The RNA pellet was dissolved in RNase free water and incubated for 10 minutes at 55^{0}C and finally quantified by using Nano drop.

***cDNA synthesis using iScript select cDNA synthesis kit*:** The cDNA were synthesized from the RNA extracted from day 1 to day 7 using iScript select cDNA synthesis kit (Bio-rad). The RNA from the cell culture supernatant sample were mixed with 4 µl of 5X iScript select reaction mix, Gene specific primer (5 picomoles/µl), GSP enhancer solution (1 µl) and Reverse transcriptase enzyme (1 µl) and finally RNase-free water was added to make the final volume to 20 µl. The program consisted of 25^{0}C for 5 minutes, 42^{0}C for 60 minutes, followed by 85^{0}C for 5 minutes. The cDNA product thus obtained was stored at -20^{0}C until further use.

***SYBR Green 1 based real time RT-PCR and Melting curve analysis*:** The real-time RT-PCR (Bio-rad) based on SYBR Green 1 (Bio-rad) was carried out using different dilutions of the cDNA (1:100 and 1:1000) product synthesized from the iScript cDNA synthesis kit. The reaction mixture consisted of 5 µl of cDNA product, 12.5 µl of SYBR Green mix, 0.5 µl of D1 primer, 0.5 µl of D2 primer and 6.5 µl of sterile H_2O to make a final volume of 25 µl. The SYBR Green 1 real time RT-PCR amplification reaction conditions includes 95^0 C for 5 min of pre incubation, 95^{0}C for 30 sec (denaturation), 60^{0}C for 30 sec (annealing) followed by 68^{0}C for 1 min (extension). The reaction continued for 29 cycles with different dilutions of cDNA (1:10 and 1:1000), and the Ct-value for each of the dengue strains were checked using amplification graphs. Since, we incorporated the SYBR Green, a non–specific probe, which binds to the amplified double stranded DNA during real-time RT-PCR, The melting curve analysis was done in the mean time to confirm the amplified product by determining its melting temperature (T_m). The melting curve analysis involves heating the amplified product to 95^{0}C for 1 min and then cooling to 60^{0}C and finally, increasing to 95^{0}C with a transition rate of 0.5^{0}C/30 sec. The samples were run on 1.5% agarose gel to confirm the specific product by size.

***Standardization and normalization of real-time RT-PCR reactions*:** The standardization and normalization of real-time RT-PCR reaction was done in two separate reaction mixture using DENV-4 and GAPDH respectively. The Vero cells

seeded at $0.1x10^6$ cells/ml were infected with DENV-4 and the 7th day supernatant collected for RNA extraction and different concentrations (Neat, 1:10, 1:100 and 1:1000) of cDNA (5 µl) was used with D1 and D2 primers and ultimately real-time RT-PCR was performed as above said. In the same way, GAPDH gene was used to synthesize the cDNA and real-time RT-PCR protocol was repeated to normalize the reaction.

Primers used for the Study:

Name of the Primer Primer sequence

D1 5'-TCAATATGCT GAAAC GCGCGAGAAACCG-3'
D2 5'-TTGCACCAACA GTCAATGTCTTCAGGTTC-3'

Results

***Primer selection using 5¹-UTR alignment of four DENV serotypes*:** Although DENV serotypes are antigenically similar, genetically vary among the serotypes and among the strains of the single serotype. Since each serotypes comprises different strains exhibiting distinct genomic variability. To this end, we aligned the 5¹-UTR of the all DENV serotypes and the primer pair common of these conserved sequences was selected. The rAdsh5b construct harboring the shRNA sequences to target the conserved sites in 5¹-NTR was utilized to combat the DENV replication (Table-1).

Specificity of real-time RT-PCR: The specificity of any primer set can be determined by the respective product obtained in an amplification of specified cDNA. It has been shown that, cDNA can be transcribed in the absence of specific primers due to the false priming of RNA during the reverse transcriptase step (27, 28). Hence, investigators have started tagging the primer ends to differentiate the correctly primed once with false primed cDNA using SYBR Green dye (29). In contrast to these, the specificity of our selected primers was verified by the threshold value of the

Table 1. The 5'UTR region of all the four DENV were aligned and set of sequences (shown in red) selected for constructing rAdsh5b were shown and the last underlined sequences(DENV-4) were more similar with the selected construct and hence is more sensitive compared to other prototypes.

	Sh-5b
DENV-1	GTTCTAACAGTTTTTT--ATTAGAGAGCAGATCTCTGATG---
DENV-2	GTTCTAACAGTTTTTT-AATTAGAGAGCAGATCTCTGATG---
DENV -3	GTGCTGACAGTTTTTT--ATTAGAGAGCAGATCTCTGATG---
DENV -4	GTTCTAACAGTTTGTTTGAATAGAGAGCAGATCTCTGGAAAA

Table 2. The specificity of the SYBR green 1 based RT-PCR assay with the selected primers was determined by conducting parallel reactions with and without primers, to check the viral spike in alternative conditions has shown the differential threshold values in both conditions.

Sample name	Den1/ VC	Without primer	Den-2/ VC	without primer	Den-3/ VC	without primer	Den-4/ VC	without primer
Ct value (1:10)	16.37	20.68	15.84	20.20	16.70	20.47	19.47	N/A
Ct value (1:1000)	19.94	18.48	19.11	24.73	19.59	20.40	19.08	22.02

corresponding real- time RT-PCR reactions involving the panel of DENV amplification with and without the primer. The threshold value was more and was nil in some reaction without the primers D1 and D2 but in the presence of the same primers the representative DENV cDNA were amplified as evidenced by the threshold value compared to the without primer value (Table-2). This strongly implies that the selected primers are specific for the selected serotypes of the DENV.

***Sensitivity of real-time RT-PCR*:** The sensitivity of the SYBR Green 1 based real-time RT-PCR was determined by the Ct-value and the melt curve for each of the DENV serotypes. The threshold value within the same DENV serotype varied widely with different dilutions (1:10 and 1:1000) and the corresponding melting curve is also depicted the decrease in the viral load upon dilution represented by the shortening of the melting peak. The DENV-4 serotype is more sensitive when compared to the other serotypes. This is because the specificity of the consensus primers D1 and D2 towards the 5¹-UTR was more for DENV-4 than other serotypes. Hence, standardization of the assay was done with DENV-4 against the mock infected sample (Fig. 3).

***The DENV viremic spike differentiation in the presence of rAdsh5b using SYBR Geen 1 based real-time RT-PCR*:** The real-time RT-PCR diagnosis of dengue based on 3¹-UTR has already been performed utilizing SYBR Green 1 dye which binds to the amplified DNA product and ultimately detects the specific dengue serotypes using melting curve analysis (21). However, there is a paucity of detection methods which are based on 5¹-UTR of representative dengue prototypes. In the present experiment, despite utilizing the real-time RT-PCR based on 5¹-UTR for detecting the dengue types, it is employed as a tool for differentiating the disease severity while treated with rAdsh5b anti-viral regimen. The complementary DNAs (cDNAs) synthesized utilizing the consensus primers D1 and D2 common to the 5¹-UTR conserved regions were used for the real-time RT-PCR in different dilutions (1:10 and 1:1000) showed characteristic differences in the Ct-value among the DENV serotypes. The threshold value (Ct) differences in the amplification graph represent the viral load in each dilution of the specific DENV serotypes (Fig.1 and 2). The amplification of specific DENV stereotypes was confirmed using melting peaks and subsequently by the gel electrophoresis. The clear band observed

Table 3. In an parallel RT-PCR reaction with DENV1, 2, 3 and 4 alone and along with rAdsh5b regimen at different dilutions (1:10 and 1:1000) have yielded the observable differences in the threshold values. The increase in the threshold value in each dilution indicates the inhibitory effect of respective rAdsh5b against all DENV selected.

Sample Name	Den-1/ VC	Den-1 /rAdsh5b	Den-2/ VC	Den-2 /rAdsh5b	Den-3/ VC	Den-3 /rAdsh5b	Den-4/ VC	Den-4 /rAdsh5b
Ct value (1:10) dilution	16.37	18.14	15.84	17.46	16.70	18.79	19.47	23.07
Ct value (1:1000) dilution	19.94	20.66	19.11	20.75	19.59	21.75	19.08	22.72

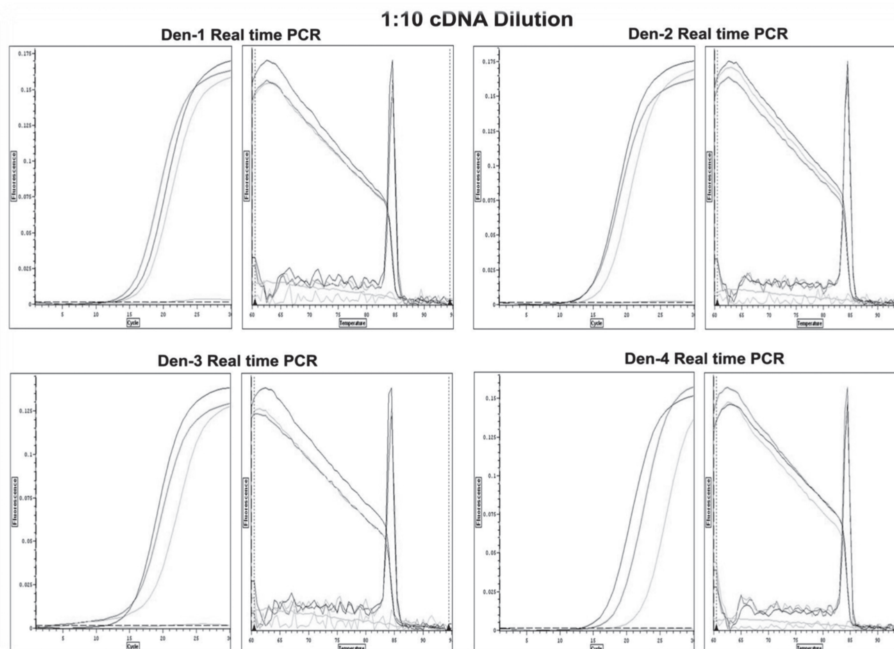

Fig. 1. The amplification graph represents the real time PCR of the 1:10 diluted cDNA of DENV1, 2, 3 and 4 and along with the rAdsh5b, rAdshN, and without primer. The graph indicates the variations in the threshold value of the rAdshN and without primer, and the rAdsh5b against each of the DENV with the fixed dilution of 1:10 of cDNA. The threshold value was more in the rAdsh5b treated sample compared to VC of each of DENV but it was less than the without primer sample implies that, rAdsh5b hindered the replication of DENV ,and hence due to the less DENV specific nucleotides, the Ct value increased significantly compared to VC.

corresponding to 512 mb band indicates amplification was specific for representative serotypes of the DENV (Fig. 4). If the copy number of the target nucleic acid is more in the sample, sooner is the threshold value and subsequent increase in the fluorescence. The threshold value increased in the rAdsh5b treated samples when compared to the each DENV serotypes in both the dilutions implies that, rAdsh5b successfully inhibited the replication of all the four DENV serotypes (Table-3).

Discussion

Since dengue serotypes exhibit variant genetic strains, many real-time RT-PCR methods have been deduced in the recent decades with the concerted effect of advanced detection chemistries and updated molecular information about the emerging dengue viruses. All the assays described in the literature so far have shown that the DENV can be detected and serotyped using real-time RT-PCR employing the primer or probes directed against the conserved regions of the dengue genome such as 3¹-UTR and 5¹-UTR (24). However, there have been efforts to utilize the RT-PCR to quantify the immunodeficiency virus type1 viral load in disease progression and as well in response to the antiviral regimen in the plasma sample (30). In contrast, this is the first report describing the utilization of real-time RT-PCR to detect the dengue serotypes whilst in the disease progression and viral load with response to antiviral regimen. In the present study we showed that SYBR Green 1 based real-time RT-PCR method utilizing the consensus primers common to the 5¹-UTR conserved sequences

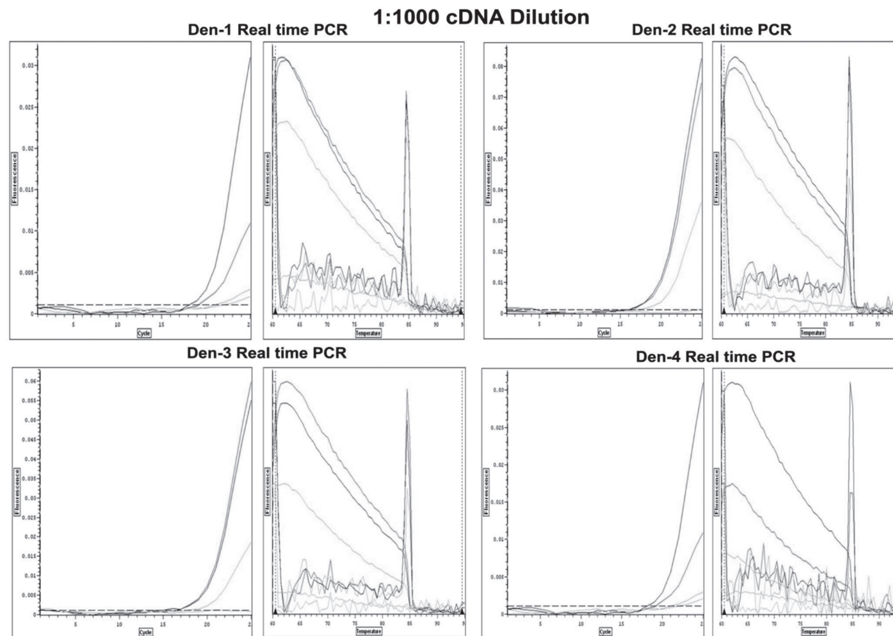

Fig. 2. The amplification graph represents the real time PCR of the 1:1000 diluted cDNA of DENV1, 2, 3 and 4 and along with the rAdsh5b, rAdshN, and without primer. The graph indicates the variations in the threshold value of the rAdshN and without primer, and the rAdsh5b against each of the DENV with the fixed dilution of 1:1000 of cDNA. The threshold value was more in the rAdsh5b treated sample compared to VC of each of DENV but it was less than the without primer sample implies rAdsh5b hindered the replication of DENV ,and hence due to the less DENV specific nucleotides, the Ct value increased significantly compared to VC. With comparison to 1:10 dilution, the Ct value in this dilution have comparably hiked indicating the efficiency detection limit of the real time PCR method with higher dilution sample selected.

can be used to detect all the four dengue serotypes and in fact, confirmed by melting peaks of RNA from the cell culture sample infected with rAdsh5b antiviral regimen. The efficiency of the real-time RT-PCR in discriminating the viremic spike in the presence and absence of antiviral molecule has proved that each DENV serotypes spiked according to their genetic variability as indicated by the threshold values. Since, threshold value depends on the compatibility of the viral RNA sequences with available primer and nucleotides, the specific viremia in the sample can be detected according to its threshold value. The sample infected with DENV 1, 2, 3, and 4 alone at different dilutions showed the lower threshold value compared to the samples infected with rAdsh5b along with all four serotypes and amplification of specific dengue serotypes in both the cases has confirmed using the melting peak shown at 84.5^{0}C which represents the 512 mb in the agarose gel electrophoresis (Fig. 4). The difference in the Ct-value is due to the fact that, rAdsh5b successfully inhibited the replication of all the dengue serotypes by targeting the 5^1-UTR regions which is common to consensus primers D1 and D2. Hence, due to the scarcity of dengue specific viral RNA in the rAdsh5b treated sample the primer spared as unused. However, there is a detectable threshold value in all the samples and in both the dilutions ascribed to the fact that the homology among different dengue genomes may decrease towards the 5^1-UTR region in the same way as reported in the 3^1-NTR (31).

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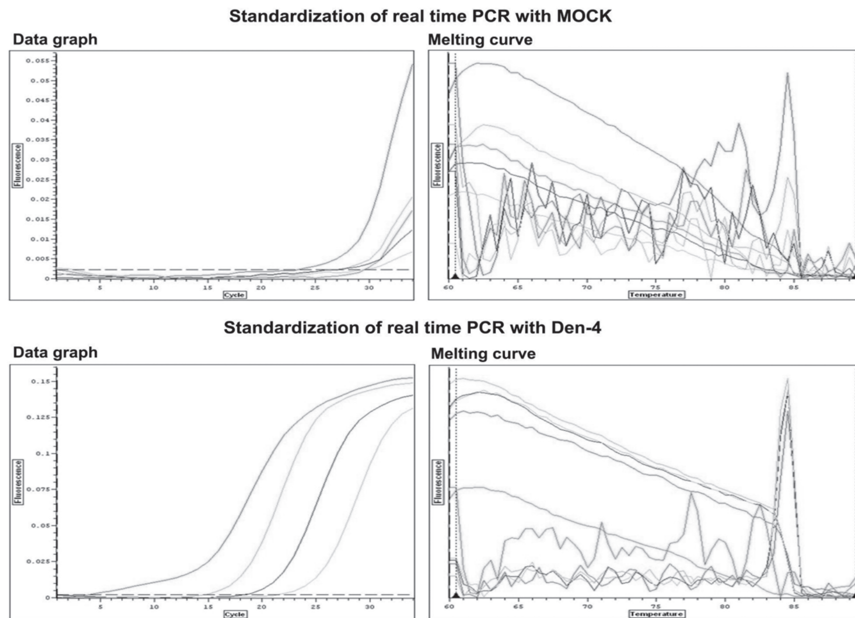

Fig. 3. The standardization of the real time PCR assay done with DENV 4 and with mock infected sample. Since, DENV 4 showed more similarity with the selected construct, the sensitivity criteria are being considered. The amplification graph obtained with the different dilutions neat, 1:10, 1:100, 1:1000 showed marked Ct values compared to the mock infected sample.

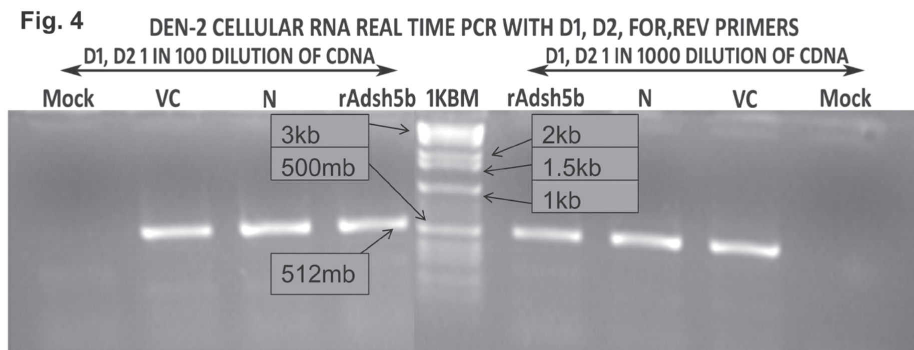

Fig. 4: The agarose gel from the melting curve analysis samples showing the clear 512 kb band indicates that, the real time PCR was specific with the SYBR green 1 dye. The single band in the gel confirms the absence of primer dimers in the reaction.

Conclusion

The results obtained from our study have explained that SYBR Green 1 based real-time PCR method can be used efficiently to detect and differentiate the viral RNA spike in the presence of antiviral regimen rAdsh5b and hence the assay can be utilized as rapid, sensitive and efficient detection method to check the dengue in the presence of antiviral regimen. Though we utilized the consensus genome coding regions of the dengue for the real-time PCR to identify the different dengue serotypes, the effort was not successful since, the homology among selected dengue serotypes was not completely conserved. This may be because of the variation in the dengue genome either due to the mutation among genotypes, and the strains within a single serotype. Hence, advanced dengue genome scanning methodologies will need to emerge in the near future to identify the most conserved sequences and as well sequences prone to mutation. This effort can be helpful to tag the primer sequences specific for conserved sites and mutation sequences to track the viral RNA degeneracy.

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Amalakirasayana feeding declines the DNA Damage in wistar rat Cerebellum, Liver and Testis, through age

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Abstract

The genomic stability has been validated in tissues of the Wistar NIN rats in terms of Single Strand Breaks (SSB) and Double Strand Breaks (DSB), by oral feeding *Amalakirasayana*, supplied to us by experts of Aryavaidyasala, kottakkal, Kerala, India, for a period of 3 and 9 months starting at the animal age of 6 months. After successful feeding, the rats were sacrificed and checked for the analysis. Making use of the enzymes E. Coli Pol I (Pol I) and Calf Thymus Terminal Transferase (TdT) the SSBs and DSBs were measured. The results were significant in the experimental rat group, compare to the control. These results convincingly indicate that, the rasayana fed animals showed significantly less number of DNA damage than in the control animals, which points out the valuable impact of rasayana therapy towards the maintenance of stable DNA damage through age. We estimate that, the distinct decrease in the DNA strand breaks of rasayana fed animal results could be because of the increased repair capacity of the damaged DNA and the anti-oxidant action of the rasayana concoction with blended additives.

Key words: Ayurveda, *Amalakirasayana,* Aging, DNA Damage, tissues

Introduction

The DNA damages every day and it gets repaired constantly by an enzyme called polymerase β(1,2). But at some point of the age the enzyme capacity declines, this decrease in the repair capacity and the building up of the damaged DNA increases in the body (3) and lowers the overall metabolic activity which leads to death. Aging is a gradual change in an organism that lowers the immunity, muscle loss, physical and mental health (4) which increase the disease causing condition and other aspects of knowledge which involves gradual molecular modifications that leads to the biochemical differences with tissue imbalance (5).

Ayurveda is one of the world's oldest holistic healing systems that developed thousands of years back in India, and now it is considered as an alternative medicine globally (6). It is believed that the health and wellness depend on a delicate balance between the mind, body, and spirit(7). Ayurvedic rasayanas are the powerful anti-oxidants and the natural healing systems which are well known to be responsible in restoring cellular equilibrium and maintain overall physiological balance(8,9).

As the scientific proof and the validations are lagging for ayurveda, it is been disregarded since decades, but now it has the surge of interest in the traditional medicine(10), of the affordable cost and the natural healing power of the rasayanas, now it is used in the practice. The concoction of the rasayana prepared by the pioneers of ayurveda from the plant extracts and

some of the herbs have a great effect in long run. One such ayurvedic rasayana, *Amalakirasayana* has been orally fed to 6 months old wistar rats of NIN for a period of 3 months and 9 months to check the DNA damage levels of Cerebellum, Liver and Testis organs of male rats with the control group. We got the significant results in *Amalakirasayana* fed animals as compare to the normal control group.

Materials and Methods

Animals: The rat experimental methods were affirmed by the 'Ethical Committee on Animal Experiments' at the National Institute of Nutrition, Hyderabad, India. 32 male wistar NIN (WNIN) rats were procured from the institute at the age of 6 months from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad. The animals were kept up as two groups; *Amalakirasayana* fed (male, $n = 16$) and Control (male, $n = 16$), maintained in polypropylene cages (two animals in each cage), The *Amalakirasayana* was fed by oral gavages to the experimental group animals at 4.5 gms per kg body wt. of the animal, along with normal given diet, and the control group animals were fed placebo (same amount of buffer used for making the rasayana suspension) with normal diet. The animals were maintained at temperature of $22 \pm 2^{o}C$, relative humidity $55 \pm 10\%$ and on standard lighting conditions (12-h light/dark cycle). Both the experimental and control animals were sacrificed at 9 months and 15 months age of the animal (which implies 3 and 9 month fed) within the stipulated time intervals and all the organs were collected and labelled including brain and they were snap frozen and stored in -80°C for further experiments.

Chemicals: *Amalakirasayana* was prepared and supplied by Aryavaidyasala, Kottakkal, Kerala, India. In brief, preparation of *Amalakirasayana* takes place in four stages; in stage 1 dried goose berry (emblica officinalis) is pulverized by using tyco pulveriser. At stage 2, fresh gooseberry juice is prepared by using a juice extractor. At stage 3, the products obtained at stages 1 and 2 are blended and dried at 55°C under low pressure of 700 mm in a vacuum tray drier. The dry mass thus formed is then pulverized and stages 2 and 3 are repeated another 20 times (Total 21 times trituration). This procedure takes about 2-3 months to complete. At Stage 4, the thick paste of *Amalakirasayana* is prepared by blending the dry powder with ghee and honey, the procedure mentioned above is adapted from the classical procedures recommended in ancient scriptures (11).

Pol I and (TdT) were purchased from Fermentas, and Thermo Fisher Scientific respectively. Calf Thymus DNA, PPO, POPOP, Unlabeled nucleotides all dNTPs were obtained from Sigma chemicals Co. (St. Louis, MO, USA). Get™ DNA template kit was purchased from G-Biosciences (St. Louis, MO, USA). 2.5cm glass fibre filter was purchased from Millipore. Radio labelled [α^{32}P]-dCTP (PLC-102) was purchased from JONAKI-BRIT (Hyderabad, India). All other chemicals used were of analytical grade. The radioactive counts were taken in Tricarb Liquid Scintillation Counter, PerkinElmer (Waltham, MA, USA).

Single and Double Strand Breaks Estimation from the tissue isolated DNA: According to the GET™ DNA Template Kit instructions the Genomic DNA from various tissues was extracted and the quantification and estimation of the DNA purity is done by using Nanodrop™2000 (Thermo Scientific, USA) at 260 and 280 nm. The single and double strand breaks assays was performed based on the ability of Pol I (As per the company instructions, 1enzyme unit catalyzes the incorporation of 10 nmol of deoxyribonucleotides into a polynucleotide fraction in 30 min at 37°C) to add nucleotides at a free 3'-OH group present in one of the duplex DNA strands, which was described earlier(12) and if the conditions are controlled so as to have a known number of nucleotides added at each of the free 3'-OH group, using the other strand as template, then it can be easy to back calculate the number of SSBs in a given sample of DNA. Assuming, each diploid cell in rat has 6 pico gm of DNA, the number of SSBs in a cell can be

calculated. Similarly the property of TdT to add nucleotides at 3'-OH group present at any blunt end of DNA without any need for a template, is exploited to assess the number of DSB in a given DNA sample and in a cell.

These assays was performed as described in our earlier paper(11) to explain in brief, for estimation of single strand breaks, the volume of the reaction is 50 μl which contained 40mM Tris-HCl(pH 8.0), 1mM β mercaptoethanol, 4mM ATP, 7.5mM $MgCl_2$, 100 μM of dATP, dTTP and dGTP and 25 μM of dCTP, 1μCi of [α^{32}P]-dCTP (3000 Ci/mmol), 1 unit of Pol I and 500 ng of genomic DNA. The reaction was carried out at 37°C for 20 min and stopped by keeping on ice and by addition of 1 ml of chilled TCA (10%) containing 10mM tetrasodium pyrophosphate. CT DNA and 200 mg of BSA were added as carriers. The samples were incubated on ice for 5 min and then centrifuged for 15 seconds at 12,000 rpm at room temperature (RT). The acquired supernatant was discarded and pellet was dissolved in 400 ml of NaOH (0.2 N). When the whole pellet was dissolved in NaOH then TCA (10%) solution (1 ml) was added to the dissolved pellet and centrifuged for 5 min at 6,000 rpm at RT. The acquired supernatant was discarded and the pellet was thoroughly resuspended in 500ml of chilled TCA (5%). The whole solution along with the precipitate was transferred onto a 2.5 cm glass fiber filters and washed six times each with chilled TCA (5%) and ethanol (95%) under a vacuum suction unit. The washed fiber filters were allowed to dry for 20 min in oven at 40°C or keeping in hood, overnight. The dried filters were taken in toluene based scintillation fluid (5 grams PPO and 0.5 grams of POPOP) per litre having 0.1% triton-X-100 and the radioactivity was counted for Disintegrations per Minute (DPM) of the individual sample with a Packard Tri-Carb Liquid Scintillation Counter.

Double Stranded DNA Breaks Estimation from the tissue isolated DNA: The estimation of double strand breaks was the similar method as in SSBs with a little change in the initial stage. The total reaction volume of 50 μl contained 100 mM sodium cacodylate buffer (pH 7.0), DTT (0.2 mM), $CoCl_2$ (1mM), 1μCi of [α^{32}P]-dCTP and 1 U of TdT (As per the company instructions, 1 U of the enzyme catalyzes the incorporation of 1 nmol of deoxythymidylate into a polynucleotide fraction in 60 min at 37°C) and 500 ng of genomic DNA. The reaction was carried out at 37°C for 30 min. The remaining procedure is the same as described for single strand breaks assay.

Statistical Analysis: Statistical analysis was performed by Graph pad Prism 5.0.3 software (Graph pad Software Inc., CA, USA). Data were expressed as mean ± standard error of mean (SEM) of three independent experiments. Significance of difference between the groups was analyzed by Student's t-test and one way ANOVA with Tukey *post hoc* test. A p-value $P \leq 0.05$ (*), and $P \leq 0.01$ (**) was considered significant for all interpretations and conclusions.

Results and Discussion

The results shown in a bar graph representation and the values on the bar graphs represent the damaged values in arbitrary units which was analysed by the graph pad prism software. In Fig 1, the single strand breaks were more in control with the damaged value of 13.6 and with an experimental value of 7.8 which shows significant at the 3 months of *Amalakirasayana* fed, when the feeding extended up to 9 months then, the results were equal in both the groups. Which implies that, even after long term feeding of the rasayana to the animals may not have effect on the DNA damage in Cerebellum, but it was not harming to the animal. In fig 2, the Liver DNA damage was much lower in the case of 3 months fed animals and significantly decreased in 9 months fed animals when compare to the control group. In Fig 3, the highly proliferating testis tissue had a significant value in the 9 months fed animals but not in 3 months fed.

When it comes to the Double Strand Breaks which are well known to be lethal to the body, have shown good effect in controlling the damages significantly, in all the tissues at 15

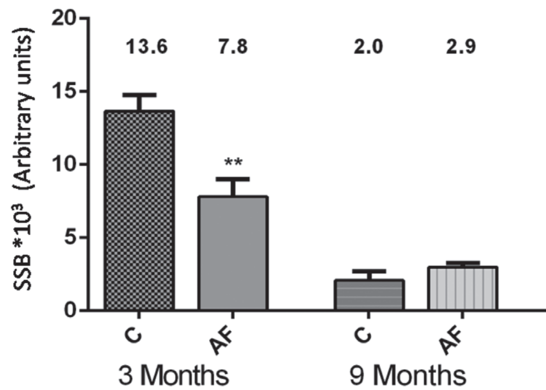

Fig. 1. Assessment of DNA-Single Strand Breaks, extracted from rat Cerebellum of control and *Amalakirasayana* fed for 3 months and 9 months through a sensitive biochemical assay which were tagged with radioactive deoxy nucleotide. ** indicates the P<0.01. This indicates that there is a significant decrease in the damaged DNA of 3 month feeding animals.

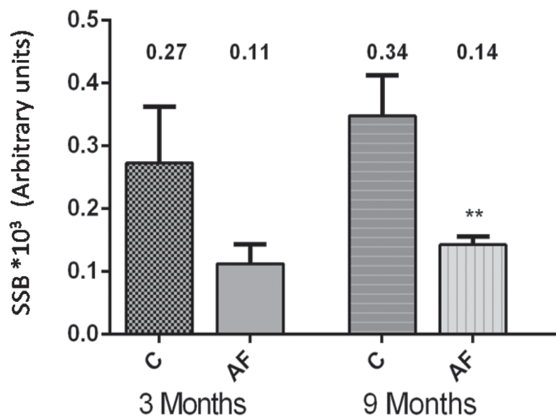

Fig. 2. Assessment of DNA-Single Strand Breaks, extracted from rat Liver of control and *Amalakirasayana* fed for 3 months and 9 months shows a significant decrease in the 9 month Amalaki fed animal's DNA. It has also the effect in 3 months but not significant.

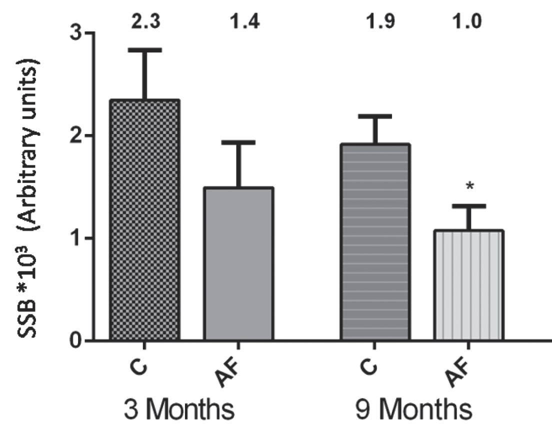

Fig. 3. Assessment of DNA-Single Strand Breaks, extracted from rat Testis of control and *Amalakirasayana* fed for 3 months and 9 months. The 9 month Amalaki fed animals has a significant decrease in the damage of DNA. And the *indicates the P<0.05.

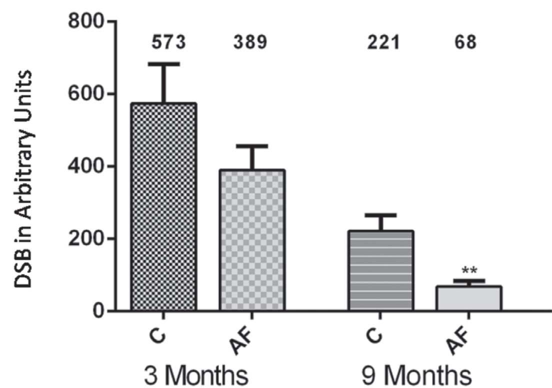

Fig. 4. DNA Double Strand Breaks of the Cerebellum extracted from the control and *Amalakirasayana* fed animals for 3 months and 9 months showing significantly decreased in the 9 months Amalaki fed. The DNA Double Strand Breaks are the distressing damages which occur in the DNA through the age. And they are even susceptible to cause DNA mutation which lead to the death of the animal.

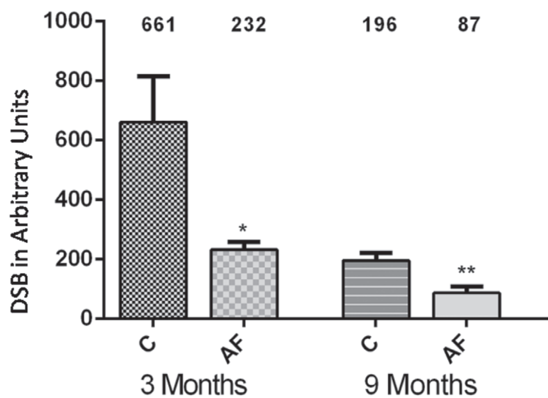

Fig. 5. DNA Double Strand Breaks of the Liver extracted from the control and *Amalakirasayana* fed animals for 3 months and 9 months showing significantly decrease in both 3 and 9 months Amalaki fed.

months aged *Amalakirasayana* fed animals (check Fig 4, 5 & 6). But in the liver tissue, Fig 5 showed significance in 3 months and as well as in 9 months *Amalakirasayana* fed animals. The observed pattern of the results thus implies that, the rasayana therapy confers significant protection against the forthcoming ailments, especially through age.

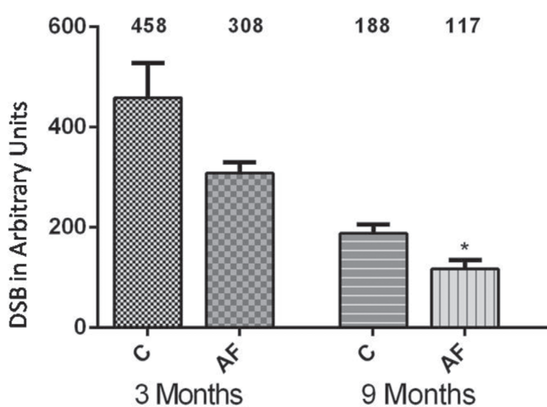

Fig. 6. DNA Double Strand Breaks of the Testis extracted from both the control and *Amalakirasayana* fed animals for 3 months and 9 months showing significantly decrease in double strand breaks in 9 months Amalaki fed animals.

Conclusions

The rasayanas are said to be more effective when they are taken in an empty stomach, which we did for the animals in our study. The results clearly explain that, upon feeding the *Amalakirasayana*, animals have showed less number of damages compare to the control in long term intake of the rasayana. The *Amalakirasayana* which was a concoction of gooseberry, honey and ghee, which are known to have abundant anti-oxidation properties and with the honey and ghee concoction taken in an empty stomach, would have enhanced the mechanism of controlling the DNA damage. We cannot estimate the root cause behind these encouraging results as of now but, the anti-oxidation property of the rasayana concoction and its ability to metabolise and maintain the healthy environment of the cell could be one of the reasons behind this rejuvenation.

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Role of Phytohormones during Salt Stress Tolerance in Plants

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Abstract

Phytohormones are chemical substances that induce physiological responses in plants in minute concentrations. Apart from the classical five phytohormones viz., auxins, gibberellins, cytokinins, ethylene and abscisic acid, there are other well-known phytohormones like brassinosteroids, methyl jasmonates, salicylic acid, strigolactones etc., that have a profound role during abiotic stress tolerance. Plants are prone to various abiotic stresses like heat, chilling, freezing, drought, flooding, oxidative, salt, allelochemicals, radiation, light, wind, heavy metals stresses etc. Among the various abiotic stresses in plants, salt stress is an important aspect that hinders growth, metabolism and final yields of plants. The roles of above mentioned phytohormones which can alleviate the salt stress are slowly unfolding. The present review deals with the role of the phytohormones on the alleviation of salt stress in plants.

Key words: Phytohormones, Salt stress.

Introduction

Plants are frequently prone to different types of abiotic stresses such as drought, chilling, heat, or salinity stress (1). Other abiotic stress factors such as cold, irradiation, or light stress are also known to adversely affect the crops (2). Salinity usually occurs through natural or human-induced processes which results in the accumulation of dissolved salts in the soil water which in turn inhibits plant growth (3). Salts in the soil water usually inhibit the metabolism by reducing the ability of the plant to take up water which is called as the osmotic or water-deficit effect. The excessive amounts of salt entered into the plant in the transpiration stream will result in the injury to cells and also drastically affect the transpiration. Soil salinity is one of the main factors that limits the spread of plants in their natural habitats and is an ever-increasing problem in arid, semi-arid and also in irrigated regions (4).

Phytohormones have an important role to play in mediating plant responses to abiotic stress. Plants have developed over the years a variety of physiological and biochemical mechanisms through which they survive under the stressful conditions (5). Phytohormones are produced naturally by plants and are essential for physiological responses in plants like formation of leaves and flowers, elongation of stems, development, ripening of fruits etc. The five classical phytohormones are auxins, gibberellins, cytokinins, abscisic acid and ethylene. Plant growth regulators are employed in the modern agriculture to boost the growth and yield of plants. The application of plant growth regulators (auxins, gibberellins, cytokinins and ethylene and several synthetic derivatives) in agriculture started in the early 1930s in the Unites States of America and the practices are still continuing world-wide. These phytohormones were used for protecting the plants against

various abiotic stresses. Among the many, the role of abscisic acid on the plants during salt stress has been extensively studied.

In the recent times, other phytohormones like brassinosteroids, methyl jasmonates, salicylic acid, and strigolactones have been shown not only to regulate plant growth and development but also to protect plants from various abiotic stresses like high temperature, low temperature, salt, high light, weak light, drought as well as flooding, heavy metals, herbicide stress, pesticide stress and even biotic stresses. The present article summarizes multiple roles of phytohormones play during salt stress tolerance in various plants.

Effect of auxins on salt stress tolerance : Auxins help plants to grow and promote the formation of apical meristems and are responsible for root differentiation. The role of auxins in overcoming various abiotic stresses has been discovered. Salinity stress can influence indole-3-acetic acid (IAA) homeostasis due to the alterations in IAA metabolism and distribution (6). It was observed that a membrane-bound NAC transcription factor NTM2 is a molecular link, and incorporates auxin signal into salt stress signaling during seed germination of *Arabidopsis thaliana*, thus providing a role of auxin in modulating seed germination under high salinity (7).

Effect of gibberellic acid (GA) on salt stress tolerance : Gibberellic acid (GA) is the phytohormone that plays pivotal roles in growth and metabolism of plants especially cell elongation. A central role for the GAs in the response to abiotic stresses viz., cold, salt and osmotic is becoming increasingly evident and reduction of GA levels and signaling has been shown to contribute to plant growth under abiotic stresses (8). Gibberellic acid (GA_3) reduced NaCl-induced growth inhibition in rice (*Oryza sativa* L. cv. Nipponbare) in a concentration-dependent manner, including the length of root tissue by regulating some salt-regulated proteins like glutamyl-tRNA reductase, enolase, salt stress-induced protein (SALT protein), a hypothetical protein OsJ_014066, putative chaperonin 21 precursor, another hypothetical protein OsJ_025258, ribulose bisphosphate carboxylase, isoflavone reductase-like protein and phosphoglucomutase, providing a new insight to reveal the modulating effect of GA_3 on salt stress in rice (9). GA_3-priming-induced increase in grain yield of two spring wheat (*Triticum aestivum* L.) cultivars, namely, MH-97 (salt intolerant) and Inqlab-91 (salt tolerant). This increase in yield is attributed to the GA_3-priming-induced modulation of ion uptake and partitioning (within shoots and roots) and hormone homeostasis under saline conditions (10). The application of GA_3 in combination with $CaCl_2$ mitigated the adverse effect of salinity stress by increasing the growth, physio-biochemical parameters, proline, glycine betaine content, activities of superoxide dismutase and catalase in linseed (*Linum usitatissimum* L.) (11). GA_3 ameliorated the adverse effects of salt stress and restored normal growth in terms of plant length and plant fresh/dry biomass and development in other crop plants like soybean also (12).

Effect of cytokinins (CKs) on salt stress tolerance : Cytokinins (CKs) are the phytohormones that regulate plant growth and development and play prominent roles in cell division via a complex network of CK signaling. CKs have been studied for their roles in mitigating various abiotic stresses in plants. The functional analyses with CK-deficient plants to provide direct evidence that CKs negatively regulate salt stress signaling was reported (13). Cytokinins play a significant role during several plant growth and developmental processes including cell division, chloroplast biogenesis, apical dominance, leaf senescence, vascular differentiation, nutrient mobilization, shoot differentiation, anthocyanin production, and photomorphogenic development (14). It was reported that it increases salinity tolerance via increased proline contents in egg plant under exogenous application of CKs (15). Principal component analysis revealed that leaf xylem cytokinins (CKs) control leaf growth and photosystem II efficiency (Fv/Fm) and thus crop

productivity in tomato plants (cv Boludo F1) grafted onto a recombinant inbred line (RIL) population derived from a *Solanum lycopersicum* x *S. cheesmaniaecross* grown under moderate salinity (75 mM NaCl) for 100 days under greenhouse conditions (16). Enhancing the root cytokinin synthesis modified both shoot hormonal and ionic status, thus ameliorating salinity-induced decreases in growth and yield of tomato (*Solanum lycopersicum* L.) plants (17).CKs and CK signaling regulated the plant adaptation to stress by His–Asp phosphorelay, an important component of the CK signal transduction pathway, triggering CK-responsive genes under salt stress (18).

Effect of ethylene on salt stress tolerance : Ethylene plays a prominent role in fruit ripening as well as senescence. The ability of ethylene to alleviate various stresses in plants is one of the main ongoing research topics. The application of ethylene improved plant salt tolerance by improving chlorophyll a/b, photosystem II function (Fv/Fm), redox state and retention of K^+ in shoots and roots of *Arabidopsis* wild type (Col-0), ethylene insensitive mutants (ein2-5 and ein3-1) and constitutive triple response mutant (ctr1-1) also (19). Ethylene plays an important role in salt-tolerant (Indent-1) and salt-sensitive (Red Ball) genotypes of tomato subjected to salt stress by regulating plant responses viz., chlorophyll content index (CCI), stomatal conductance and ion homeostasis (20).

Effect of abscisic acid (ABA) on salt stress tolerance : Abscisic acid (ABA) is a sesqui terpenoid phytohormone discovered in 1960s for its role in promoting leaf abscission-and seed dormancy. ABA plays a major role during many stages of the plant life cycle, including seed development and dormancy, and mediates plant responses to various environmental stresses and also acts as endogenous signal molecules responsible for inducing abiotic stress tolerance in plants (21). ABA has been proposed to play an important role in stress responses and/or adaptation (22). Under saline conditions, there is a rapid and significant accumulation of ABA which is crucial to plant protective mechanisms (23). The biosynthesis and redistribution of ABA is one of the fastest responses of plants to abiotic stresses including salt stress, causing stomatal closure, thereby reducing water loss via transpiration and eventually restricting cellular growth (24). Two varieties of maize, SRO3 (salt resistant) and Lector (salt sensitive) exhibited significantly increased concentrations of ABA (25). Exogenous application of ABA at 100 µM prior to and during the salt-stress period induced salt tolerance in both the salt-susceptible (LPT123) and the genetically related salt-resistant (LPT123-TC171) rice lines. It enhanced the survival rate and triggered proline (an osmoprotectant) accumulation earlier than that by salt-stress alone, supporting a role for induction of proline biosynthetic pathway gene expression in proline accumulation (26). ABA plays an important role in salt-tolerant (Indent-1) and salt-sensitive (Red Ball) genotypes of tomato subjected to salt stress by regulating plant responses like chlorophyll content index (CCI), stomatal conductance and ion homeostasis (20). ABA is a key endogenous messenger in plant responses to salt stress and it unfolded a unique hormone perception mechanism where binding of ABA to the ABA receptors RCARs/PYR1/PYLs lead to inactivation of type 2C protein phosphatases such as ABI1 and ABI2 and these protein phosphatases seem to function as coreceptors and their inactivation launches SNF1-type kinase action which targets ABA-dependent gene expression and ion channels (27). Thus, ABA plays a vital in transducing the signals and in triggering the downstream responses.

Effect of brassinosteroids (BRs) on salt stress tolerance : Brassinosteroids (BRs) are a new type of polyhydroxy steroidal phytohormones with significant growth-promoting influence (28). Mitchell and his co-workers (29) discovered BRs, but were later extracted from the pollen of *Brassica napus* L. (30). The widely used bioactive BRs, brassinolide (BL), 24-epibrassinolide (24-EpiBL), 28-homobrassinolide (28-HomoBL),

castasterone (CS) and 24-epicatasterone (24-EpiCS). BRs played important roles in monitoring the stress-protective properties in plants against a number of abiotic stresses like low temperature/ chilling, /freezing, salt, high temperature/heat stress, water/drought/water logging, heavy metals and biotic stresses (31). BRs confer salt tolerance to plants by mitigating its negative effects on the physiological, biochemical and molecular processes in plants (32). Exogenous application of BRs offered tolerance to salinity by altering stress responses in rice variety Pusa Basmati-1 (33). BRs enhanced the physiological mechanisms for enhancing the salt tolerance in oilseed rape plants (34). Further, the ameliorative effect of BRs on germination of cucumber seeds in the presence of sodium chloride was reported (35). The alleviating effects of brassinolides (BLs) on cucumber seedlings grown under NaCl stress was also studied (36). BLs improved the physiological characteristics of rice (*Oryza sativa* L.) under different salinity levels (37). BL improved the growth, yield and chemical composition of berseem (*Trifolium alexandrinum* L.) grown in saline soils (38). Further, it was reported that BL not only alleviated the salt stress but also increased the antioxidant activity of cowpea plants (*Vigna sinensis*) subjected to saline conditions (39).

24-epibrassinolides (24-epiBL) enhanced the physiological and genetic changes in two varieties of pepper under salt stress conditions (40). They have increased the growth and alleviated the deleterious effects induced by salt stress in pea (*Pisum sativum* L.) and treatment with 5 μM epiBL detoxified the stress generated by sodium chloride and significantly improved the growth, the level of pigment parameters, green pod yield and pod protein in *Phaseolus vulgaris* L. (41-42). 24-EpiBL regulated photosynthesis, antioxidant enzyme activities and proline content of *Cucumis sativus* under salt stress (43-44). Further, it was reported that supplementation of 24-EpiBL trigger physiological and biochemical responses for the salt stress mitigation in *Cucumis sativus* L. (45). Seed treatment and foliar application of 24-epiBL to lettuce (*Lactuca sativa* L. var. Crispa) mitigated the negative impact of salt stress by enhancing the growth, chlorophyll and mineral contents (46). 24-epiBL application ameliorated the salt-induced oxidative stress in eggplant (47) and also the adverse effects of salt stress on the stomatal conductance, membrane permeability, leaf water content, and ionic composition in strawberry (48). 24-EpiBL also restored the nitrogen metabolism of pigeonpea under saline stress (49). It played a pivotal role in pea protein tyrosine phosphorylation after salinity treatment (50) and alleviated the salt-induced inhibition of productivity by increasing nutrients, compatible solute accumulation as well as enhancing antioxidant system in wheat (*Triticum aestivum* L.) (51). Supplementation of 24-epiBL played pivotal roles on the hormonal status of wheat plants under sodium chloride stress (52).

28-homoBL alleviated the negative impact of salt stress on *Vigna radiata* by enhancing the rate of photosynthesis, fluorescence and antioxidant system (53). Foliar application of 28-homoBL mitigated salinity stress in *Brassica juncea* by increasing its photosynthetic efficiency (54). BR-analouges improved the genotypes of *Oryza sativa* L. grown under salinity stress (55). It was reported that a BR-analogue prevented the negative effect of salt stress on ethylene synthesis in lettuce plants (56). However, the exact mechanism how epibrassinolides or 28-homobrassinilides alleviate the salt stress and improve the antioxidant capacity of plants under salt stress is not clear.

Effect of jasmonates (JAs)/methyl-jasmonate (MeJA) on salt stress tolerance *:* Methyl jasmonate (MeJA) or its deesterified acid, jasmonic acid (JA) is a lipid-based hormone that regulates a wide spectrum of processes in plants, ranging from growth and photosynthesis to reproductive development. In particular, JAs play pivotal roles for plant defense against herbivory and plant responses to poor environmental conditions and other kinds of abiotic and biotic challenges. Foliar application of methyl-

jasmonate (MeJA) mitigated the salinity stress of broccoli plants (*Brassisca oleracea* L. var. Italica) by enhancing plant dry weight, leaf CO_2 assimilation, and root respiration (57). However, themolecular events leading to salt stress in presence of MeJA or JAs have not yet been elucidated.

Effect of salicylic acid (SA) on salt stress tolerance : Salicylic acid (SA) acts as endogenous signal molecule that is responsible for inducing abiotic stress tolerance in plants. It plays a major role in the regulation of plant growth, development, and interaction with other organisms and defense responses to environmental stresses (21, 58-59). It was observed that, SA is not essential for germination under normal growth conditions, but it plays a promotive role in seed germination in *Arabidopsis thaliana* under high salinity by reducing oxidative damage (60). Exogenous application of SA led to increased salt tolerance in seedlings of pistachio (59). When mungbean (*Vigna radiata* L.) cultivar Pusa Vishal plants grown with 50 mMNaCl were sprayed with 0.1, 0.5, or 1.0 mM SA, it mitigated the negative impact of salt stress by increasing nitrogen, phosphorous, potassium (N, P, K), and Ca^{2+} content, and increased the activities of antioxidant enzymes, glutathione content, photosynthesis, and final yield (61). Application of SA mitigated the negative effect of salt stress in plants and improved the physiological, biochemical and molecular events associated with plant growth and development (32). Supplementation of SA improved the biochemical parameters of wheat (*Triticum aestivum* L.) subjected to salinity stress (62). Ameliorative effects of SA in mitigating the phytotoxicity of NaCl stress in *Zea mays* L. seedlings by regulating growth traits, content of photosynthetic pigments, proline, relative water content (RWC), electrolyte leakage percent (EC%), antioxidative enzymes and leaf anatomy (63) were noticed.Exogenous application of SA to pearl millet (*Pennisetum glaucum* (L.) R. Br.) seedlings alleviated salt stress by increasing the length (plant and root), fresh and dry weights as well as glycine betaine and total soluble carbohydrates (64). Application of SA to cucumber seedlings protected them from NaCl-induced stress by increasing the levels of sugars like glucose, fructose, raffinose and stachyose which act as osmotic agents or nutrients and as metabolic signals (65). It was reported that 0.1, 0.5, and 1.0 mM SA alleviated 50 mMNaCl-induced salinity stress in two cultivars of mustard (*Brassica juncea* L.) viz., *Alankar* (salt-tolerant) and *PBM16* (salt-sensitive) plants by increasing nutrients content, photosynthetic and growth characteristics, and activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) (66). SA mitigated the toxic effect of salinity stress on tomato (*Lycopersicum esculentum* Mill.) by regulating sugar, protein and proline contents (67). However, the molecular events leading to the mitigation of salt stress has not yet been unraveled.

Effect of strigolactones (SLs) on salt stress tolerance : Strigolactones (SLs) were originally isolated from plant root exudates as germination stimulants for root parasitic plants of the family Orobanchaceae, including witchweeds (*Striga* spp.), broomrapes (*Orobanche* and *Phelipanche* spp.), and *Alectra* spp., and so were regarded as detrimental to the productivity of plants. SLs are suggested to have other biological functions in rhizosphere communications and in plant growth and development. It was proposed that plants increased strigolactone production in arbuscular mycorrhizal (AM) symbiosis which alleviated salt stress in lettuce plants by increasing plant biomass, stomatal conductance and efficiency of photosystem II (68). It was reported that stigmasterol applied as seed treatment not only alleviated the drastic effect of salt stress but also improved the quality and yield in flax plants (69). SL positively rescued *Arabidopsis thaliana* SL-deficient and SL-response mutants subjected to high salinity stress by increased leaf stomatal density relative to wild-type and slower abscisic acid (ABA)-induced stomatal closure (70).

Conclusions

Salinity is one of the major abiotic stresses that limit plant growth and productivity in many areas of the world due to increasing use of poor quality of water for irrigation. Plant adaptation or tolerance to salinity stress involves complex physiological, metabolic, and molecular pathways or gene networks. It is an established fact that phytohormones under salinity stress play critical roles in modulating physiological responses that eventually lead to the adaptation of plants. However, it is not clear how different phytohormones alleviate the salt stress in plants. We need to develop hormonal mutants for all different classes and then only it would be possible for us to unravel the mechanisms associated with salt stress alleviation.

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Syphilis Diagnosis Using an Advance Concept for Non-Treponemal Test Development

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Abstract

Today, different laboratory techniques are available for syphilis diagnosis. Among all, serological tests are the most popular because of their ease of use. Serological tests for syphilis diagnosis can be grouped into non-treponemal and treponemal types. The non-treponemal test type includes Venereal Disease Research Laboratory (VDRL) slide test, Unheated Serum Reagin (USR) test, Rapid Plasma Reagin (RPR) test and Toluidine Red Unheated Serum test (TRUST). All detects non-treponemal antibody and were introduced many years back. Still they are use for the syphilis screening in India. Treponemal test detects treponemal specific antibody and are available in different assay formats. Recently, introduction of automated treponemal test had changed the practice for the syphilis diagnosis. Many laboratories are now following reverse practice; automated treponemal test is use first for syphilis screening. Non-treponemal tests are not available in automated format. However, their availability can be very useful in syphilis diagnosis. It is ideal for the huge number of sample study especially in blood bank settings where work load is more. In current study, an attempt was made to develop a non-treponemal test which can be automated. Cardiolipin antigen was modified and coated on microwell surface through an advance concept of covalent attachment. Such antigen coated plate was studied with few syphilis reactive samples in enzyme immunoassay (EIA) format. It was found out that cardiolipin got attached to microwell surface successfully and showed promising result with end-point titer determination study with syphilis reactive samples. RPR was used as reference test. Both tests demonstrated similar result in their ability to determine the end-point titer. Further study is required with more number of samples to validate test performance for syphilis diagnosis. Described test can be useful as substitute for currently used non-treponemal tests.

Keywords: Syphilis,VDRL,USR,RPR,TRUST, EIA

Syphilis is a disease caused by a spirochete bacterium *Treponema pallidum* subspecies *pallidum.* Major route of transmission is through sexual contact. However, it may also transmitted by other modes which includes vertically from mother to fetus during pregnancy or at birth resulting into congenital syphilis, through blood transfusion and non-sexual contact (1). Therefore, syphilis infection can be either acquired or congenital type. Syphilis presents different clinical signs and symptoms. It can be classified as primary, secondary, latent, and late or tertiary (2).

The causative agent of syphilis cannot be grown on artificial culture media (3). This makes difficult in bacterial identification and characterization through routine microbiological culture techniques. However, many methods are available for syphilis diagnosis which includes

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dark field microscopy, serological tests and direct antigen detection tests (4). Among all, serology is the most frequently used method to diagnose syphilis (3, 5). Non-treponemal serological tests like VDRL, USR, RPR and TRUST have been used for syphilis diagnosis since long. Different treponemal specific serological testes are also available.

It includes *T.pallidum* Particle Agglutination Assay (TP-PA), *T.pallidum* Hemagglutionation Assay (TPHA), Fluorescent Treponemal Antibody Absorption Assay (FTA-ABS) and Enzyme Immunoassay (EIA) that detects IgG or IgM or both classes of antibody to treponemal proteins. Treponemal tests are available in automated format which is an added advantage over the non-treponemal test. Due to this, many laboratories had changed their practice to use treponemal test first for sample screening (6). Both non-treponemal and treponemal tests are useful for syphilis diagnosis. Though, both have their own limitations for syphilis diagnosis.

Antigen preparation of non-treponemal tests uses natural or synthetic cardiolipin along with lecithin and cholesterol. This antigen preparation reacts with non-treponemal antibodies from syphilitic patients and shows flocculation. Advantages of this type of test include easy to use, economical, rapid resultand easy to monitor efficacy of treatment (4). However, there are some conditions like autoimmune diseases, pregnancy, viral infections which may give false positive test result (4). Therefore, a reactive test result always need to be confirmed with a treponemal test as per traditional algorithm for syphilis diagnosis (7). Still all non-treponemal tests are being performed manually as none of them are available in automated format which serves as their limitation over the automated treponemal assays.

Many researchers tried to develop non-treponemal EIA system due to its potential for automation. Such type of system is suitable for large scale sample study. An EIA system capable of detecting IgG and IgM classes of antibody to VDRL antigen was reported in earlier study which had claimed its sensitivity and specificity equivalent to traditional non-treponemal tests (8). A urease enzyme based assay system for the detection of IgG class of antibody to VDRL antigen was also reported. This system demonstrated sensitivity and specificity of 96.6% and 99.6% respectively (9). Both EIA systems were developed with VDRL antigen which was dried on microwell surface and subsequently used in assay development. A different concept was reported in which cardiolipin antigen was modified and attached to wide varieties of molecules like KLH, BSA, IgY, synthetic proteins, biotin, streptavidin or avidin (10). This is a modern concept for cardiolipin modification and its attachment to different bio-molecules or to the solid support directly. Cardiolipin-protein or cardiolipin attached directly to microwell can use for non-treponemal antibody detection. This method fixes antigen on microwell surface very strongly through covalent linkage and can give consistent test result.

Oxidized cardiolipin preparation was obtained from Dr. Castro (CDC) for evaluation and study (10). This antigen was attached directly to microwell plate with primary amine group on it. Such type of plate can be purchased commercially from different suppliers like Biomat (Italy) and Becton Dickinson (USA). Cardiolipin was attached to microwell by following similar method that was used originally for conjugating it with protein (10). Cardiolipin coated plate was tested later (8). Tetramethylbenzidine was used as chromogen in testing protocol due to its better sensitivity over o-phenylenediamine (11). Reaction was stopped with sulfuric acid (8) and read at 450 nm wavelength. A strong reactive sample (RPR titer of 128) and a sample with moderate reactivity (RPR titer of 8) were used for testing. Each syphilis reactive sample was diluted serially in normal human serum to two fold and tested. A syphilis non-reactive sample was also run as a negative control in this study. Obtained response of samples with non-treponemal EIA was shown in figure 1.

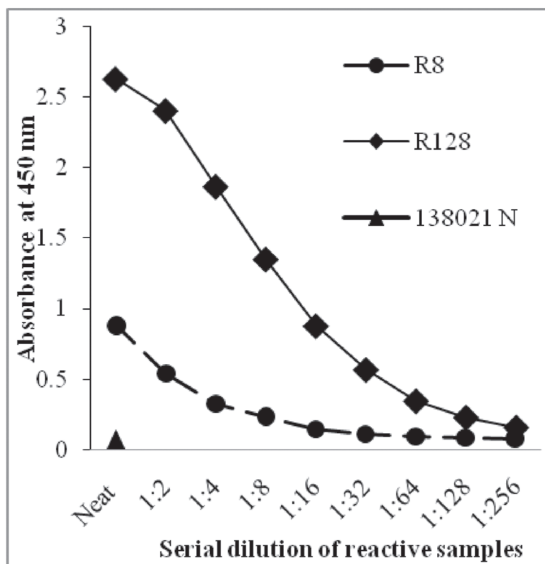

Fig. 1 End-point titer determination study with two syphilis reactive samples in developed non-treponemal EIA test.

Response of tested syphilis reactive samples with cardiolipin coated microwell demonstrated that cardiolipin got attached to microwell successfully. Decrease in absorbance was observed when syphilis reactive sample was diluted serially and tested (as shown in Fig. 1). Syphilis reactive samples were tested in RPR test kit before (ASI, USA) to determine their end-point titer. Testing was done as per manufacturer's instructions for semi-quantitative assay. The Result of non-treponemal EIA and RPR test were found out to be similar in their ability to determine end-point titer of studied reactive samples. Non-reactive sample (138021N) remained non-reactive with both tests.

In summary, sero-diagnosis of syphilis involves testing with non-treponemal and treponemal tests. Selection of a screening test depends upon acceptance of practice for syphilis diagnosis. Traditional practice uses non-treponemal test first for syphilis screening and a treponemal test later as confirmatory test. In contrast to that, reverse practice uses treponemal test first for screening and a non-treponemal test later for confirmation.Though, reverse practice was reported to yield more false positive test result than the traditional practice when it was studied with low-prevalence syphilis population (12). Treponemal antibody remains present even after successful treatment and which may give reactive test result with treponemal specific tests (5). Despite that, high sensitivity and specificity of treponemal tests for syphilis diagnosis was reported (3).

Described study demonstrated promising result with cardiolipin coated microwell prepared though an advance concept of covalent coupling. Antigen was attached to the plate surface via its tail. This may expose the head group of antigen molecule so that non-treponemal antibody can recognize it well. This enzyme immunoassay has potential for automation and may be very useful in order to satisfy the long-term need for the automated non-treponemal test. Further study is required with more number of samples with and without syphilis in order to validate the performance of this concept.

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Production and Purification of Alkaline Fibrinolytic Enzyme from *Bacillus cereus* GD55 under Solid State Fermentation and Screening for Industrial Applications

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Running title – Production of alkaline fibrinolytic enzyme

Abstract

Enzymes are well known biocatalysts that execute a multitude of chemical reactions in the metabolism of almost all organisms. From an industrial perspective, only a restricted number of enzymes are commercially available and a few of them have found applications in large quantities. Microbial fibrinolytic enzymes are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzyme technology. Alkaline fibrinolytic enzyme produced under solid state fermentation by *Bacillus cereus* GD55 was purified by ammonium sulphate fractionation (70%) and subjected to chromatographic methods. The purified enzyme produced 11.59 fold with recovery 6.8% by DEAE-Sephadex A-50 column chromatography and molecular weight was estimated to be 31 kDa by SDS-PAGE. This enzyme exhibited good compatibility with the commercial detergent Rin. The fibrinolytic potential of the enzyme was evidenced by the complete removal of blood stains from cotton cloth within 50 min of treatment. *Bacillus cereus* GD55 alkaline fibrinolytic enzyme with a diversity of activities, has great potential for application in a wide range of industry.

Keywords: Fibrinolytic enzymes, *Bacillus cereus* GD55, Solid state fermentation, Blood stains, Detergent compatibility

Introduction

Enzymes are delicate protein molecules necessary for life. Proteolytic enzymes are ubiquitous in occurrence, been found in all living organisms, and are necessary for cell growth and differentiation (1). Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers (2). Of these, strains of *Bacillus sp*. dominate the industrial sector (3). Fibrinolytic enzyme is well known as a sub class of protease, which has an ability to degrade fibrin (4-8). Fibrinolytic enzymes are the single class of enzymes, which play an important part in the metabolism of many species of *Bacillus* (9-15). The proteases are commercial value and find multiple applications in detergents, leather industry and bioremediation processes (16). The largest application of protease is in detergent industries, as they could remove protein based stains from cloths (17). For use in detergent formulations, the enzyme must be stable in surfactants, bleaching agents, fabric softeners and various other formulation aids.

Proteases are used in stiff and dull gum layer removal from raw silk to improve the softness of the silk. Wool and silk fibers will get a unique finishing when treated with protease. Removal of silver from X-ray films by decomposing the gelatin layer on the films can

be achieved by proteases (18). This enzyme can also use in contact lens cleaner which is important in bio pharmaceuticals (19). Proteases are also involved in removing necrotic material and help in natural healing (20). Chicken feather is an important poultry waste which contains 90% of protein as keratin. Biological treatment of feather is increased over the past few decades due to environmental awareness (21).

Solid state fermentation (SSF) was chosen for the present research because it shows many advantages than that of submerged fermentation, such as high volumetric productivity, use of simple machinery, use of inexpensive substrates, simpler downstream processing and lower energy requirements. (22-23). Application of SSF process has a considerable economical potential in the food, feed, pharmaceutical and agricultural industries (24-25). There are a few number of literatures reported to use the SSF process for producing fibrinolytic enzymes with industrial importance such as nattokinase, streptokinase and urokinase (23,26).

Extracellular proteases produced by *Bacillus* species are of main interest from a biotechnological viewpoint, and are not only in scientific fields of protein chemistry and protein engineering but also in applied fields such as foods and pharmaceutical industries. The genus *Bacillus* contains a number of industrially important species and approximately half of the present commercial production of bulk enzymes derives from the strains of *Bacillus*. In this study, an attempt was made for the production, purification of alkaline fibrinolytic enzyme under solid state fermentation by *Bacillus cereus* GD55 and screening for removal of blood stains and compatibility with detergents.

Material and Methods

***Experimental chemicals*:** Apple pomace was obtained from a local apple juice concentrate company in Bangalore, Karnataka, India. The substrate was washed thoroughly with tap water and then dried in an oven at 60°C. The dried materials obtained is milled and sieved to powder for using as a carbon source for fibrinolytic enzyme production. Fibrin, agarose, acrylamide, bis-acrylamide, sodium dodecyl sulfate, TEMED, ammonium persulphate and protein markers were purchased from Sigma Chemicals Co.USA. All other chemicals and analytical reagents were purchased from Hi-media, India, unless stated otherwise.

***Bacterial strain and culture conditions*:** The strain of *Bacillus cereus* GD55 used in the experiment was previously optimized for fibrinolytic enzyme production by submerged fermentation and solid state fermentation (23,27,28) and purified in our laboratory from submerged fermentation process (28,29). The isolate was grown in nutrient broth medium contained (g/l) of fructose 1, fibrin 0.2, peptone 1, K_2HPO_4 0.2, $CaCl_2$ 0.04, NH_4NO_3 0.5, $MgSO_4$ 0.02, biotin 0.05, serine 0.01, $CaCO_3$ 0.01 with 70% glycerol. Cultures were preserved at -20°C (30). The inoculum was prepared by transferring a loopful of stock culture in to 100 ml of sterile nutrient broth stock medium, then incubated it overnight at 37°C on a rotary shaker with 200 rpm, before being used to inoculation (31). A stock suspension was prepared and adjusted to 7×10^3 cells/ml.

***Production of fibrinolytic enzyme*:** SSF was carried out in Erlenmeyer flasks (250 ml) containing 10 g substrate with 10 ml of salt solution (g/l) K_2HPO_4 0.2, $CaCl_2$ 0.04, NH_4NO_3 0.5, $MgSO_4$ 0.02, biotin 0.05, serine 0.01, $CaCO_3$ 0.01, pH 8.0 and each flask was covered with hydrophobic cotton and autoclaved at 121°C for 15 min. After cooling the flasks to room temperature, the flasks were inoculated with 2 ml of 24 h grown spore solution (2×10^6 CFU/g) under sterile conditions. The moisture content of the medium was maintained at 70%. The contents of the flasks were well mixed and incubated at 37°C for 72 h in an incubator shaker at 200 rpm. At the end of fermentation, cultures were extracted with 100 ml of distilled water by shaking for 2 h. The filtrate obtained was centrifuged at 10,000 rpm for 10 min at 4°C and

the final clear supernatant (crude enzyme) was subjected to recovery and purification process (32).

***Fibrinolytic enzyme activity determination*:** Fibrinolytic enzyme activity was assessed by the modified procedure of Greenberg (1957) (33) using 2% bovine fibrin in 0.05 mM phosphate buffer (pH 8) as a substrate. Fibrin solution (0.5 ml) with an equal volume of suitably diluted enzyme solution was incubated for 10 min at 37°C. After 10 min, the reaction was terminated by the addition of 1 ml of 10% cold tri chloroacetic acid (TCA). The mixture was centrifuged at 3,000 rpm for 10 min. To the acid soluble supernatant 5 ml of 0.44 M Na_2CO_3 was added, followed by 1 ml of two fold diluted Folin-Ciocalteau reagent. The mixture was incubated for 30 min at 37°C and the colour developed was read at 660 nm against a reagent blank prepared in the same manner. Tyrosine served as the reference standard. The optical density of these solutions was measured in a Shimadzu (Japan) spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that liberated one ìg of tyrosine per ml per min.

***Total protein concentration determination*:** Lowry method was carried out by using bovine serum albumin (BSA) as standard. Serum Albumin solution was prepared in increasing concentration for the Lowry assay standard curve. The total protein concentration was determined by using spectrophotometer at the wavelength of 680 nm. Various protein concentrations were determined based on the standard curve (34).

***Ammonium sulphate fractionation and dialysis*:** All purification steps were performed at 4°C. The crude enzyme was subjected to ammonium sulphate fractionation, at different concentrations ranging from 30-80% saturation (35). The precipitates so obtained were suspended in cold saline and tested for fibrinolytic activity. The salting out concentration of crude enzyme was at 70% on the basis of enzyme activity. To obtain complete precipitation of the crude enzyme, the remaining harvest fluid was subjected to ammonium sulphate precipitation at 70% saturation. The precipitate so formed was separated by centrifugation (10000 rpm) for 15 min., again suspended in cold saline solution (100 ml) and dialyzed (36) in cold against one litre of 0.05 mM phosphate buffer, pH 8 for 24 h. After dialysis, the solution was centrifuged and supernatant obtained was used for gel filtration chromatography.

Gel filtration chromatography on Sephadex G-50: Gel filtration chromatography was carried out using a Sephadex G-50 Column (120 cm×1.0 cm) (37). The column was equilibrated with 10 mM Tris-HCL buffer, pH 8 (38). The flow rate was 1 ml/6 min. The fractions collected were determined for its total protein concentration and fibrinolytic enzyme activity. The active fraction obtained was pooled together, concentrated by lyophilization and used as purified fibrinolytic enzyme for subsequent studies.

***Anion exchange chromatography on DEAE Sephadex A-50*:** The dialyzed enzyme was chromatographed on a column of DEAE Sephadex A-50. The sample was loaded on to a column of DEAE Sephadex A-50 (24 cm× 2.0 cm) equilibrated with 20 mM Tris-HCL buffer, pH 8 (38). The absorbed protein solution was eluted at a flow rate of 1 ml/6 min with a discontinuous gradient ranging from 0.1 M to 1.0 M of NaCl dissolved in same buffer. Major peaks of fibrinolytic activity were observed in fractions 42 to 49 (FA-I), 53 to 62 (FA-II) and fraction 65 to 78 (FA-III). The fractions collected were determined for its total protein concentration and fibrinolytic activity. The active fractions 42 to 49 (FA-I) with high fibrinolytic activity were dialysed and used for further studies.

***Electrophoretic analysis*:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 5% stacking gel and a 12% polyacrylamide resolving gel according to the method of Laemmli (39). A low molecular weight marker (Bio-Rad) was used as reference proteins. Gel was stained with

Coomassie Brilliant Blue R-250 and de-stained with a solution containing methanol: glacial acetic acid: distilled water = 1:1:8 (v/v).

Enzyme stability in the presence of detergents: The compatibility of fibrinolytic enzyme with local laundry detergents was investigated in the presence of 10 mM $CaCl_2$. The following detergents were used: Nirma, Henko, Surf excel, Wheel, Rin and Ariel. The detergents were diluted in distilled water (0.7% w/v), incubated with 0.1 ml of enzyme for 60 min at 37°C and the residual activity was determined (40). The enzyme activity of a control sample (without any detergent) was taken as 100%.

Assessment of stain removal activity: Fibrinolytic activity was performed according to the method of Najafi and Deobagkar, (2005) (41). A clean piece of pure white cotton cloth was soaked in animal blood (0.1 ml) for 15 min and then allowed to dry at 80°C for 5 min in hot air oven. Then the cloth pieces were soaked in 2% (v/v) formaldehyde for 30 min and rinsed with water to remove excess formaldehyde. The dried cloth was cut into equal sizes (4×4 cm) and incubated with a crude enzyme (2 ml) at 37°C for different incubation periods (10, 20, 30, 40 and 50 min). After a given incubation, the cloth was rinsed with tap water for 2 min without scrubbing and then dried in the open air. Visual examination of various pieces exhibited the effect of enzyme in the removal of strains. The same procedure was done with the control without the enzyme exposure.

Results and Discussion

***Gel filtration chromatography on Sephadex G-50*:** The crude broth obtained after fermentation was subjected to ammonium sulphate precipitation at 70% (w/v). The pellet so obtained was resuspended in cold saline (2 ml) and dialysed. The dialysed enzyme was loaded on a column of Sephadex G-50 (120 cm × 1.0 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8. The column was eluted at a flow rate of 1 ml/6 min. The elution profile of gel filtration chromatography is shown in the (Fig: 1). The fractions collected were determined for its total protein concentration and fibrinolytic enzyme activity. The active fraction obtained was pooled together, concentrated by lyophilization and used as purified fibrinolytic enzyme for subsequent studies. The summary of purification steps involved for fibrinolytic enzyme is presented in the Table: 1.

***Anion exchange chromatography on DEAE Sephadex A-50*:** The dialyzed enzyme was chromatographed on a column of DEAE Sephadex A-50. The sample was loaded on a column of DEAE Sephadex A-50 (24 cm × 2.0

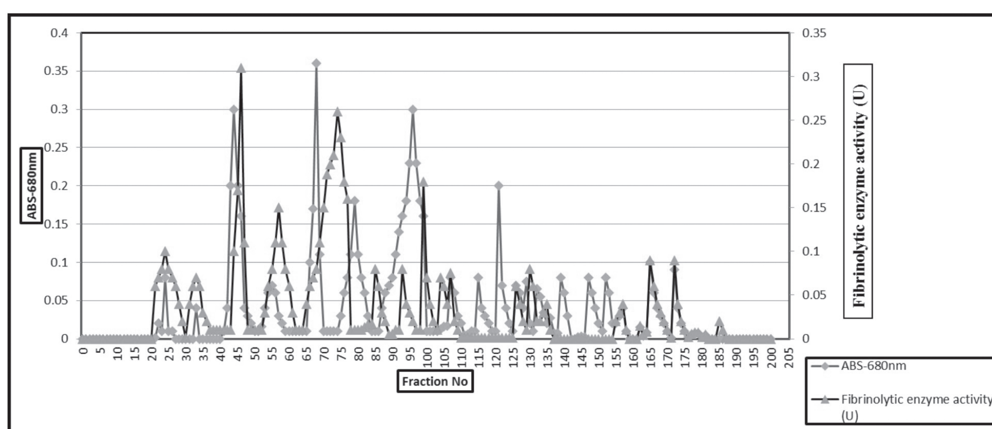


Fig 1. Gel filtration chromatography on Sephadex G-50

Table 1. Summary of the purification of alkaline fibrinolytic enzyme from *Bacillus cereus GD55*

Purification Step	Total Protein (mg)	Total activity (U)	Specific activity U/mg	Fold purification	% Yield
Crude extract	490.7	97,270	198.2	1.00	100
70% Ammonium sulphate saturation	19.4	21,229	1,094.3	5.52	21.8
Dialysis	10.8	13,351	1,236.2	6.23	13.7
Sephadex G-50	5.24	8,913	1,700.9	8.57	9.2
DEAE- Sephadex A-50	2.86	6,581	2,301.0	11.59	6.8

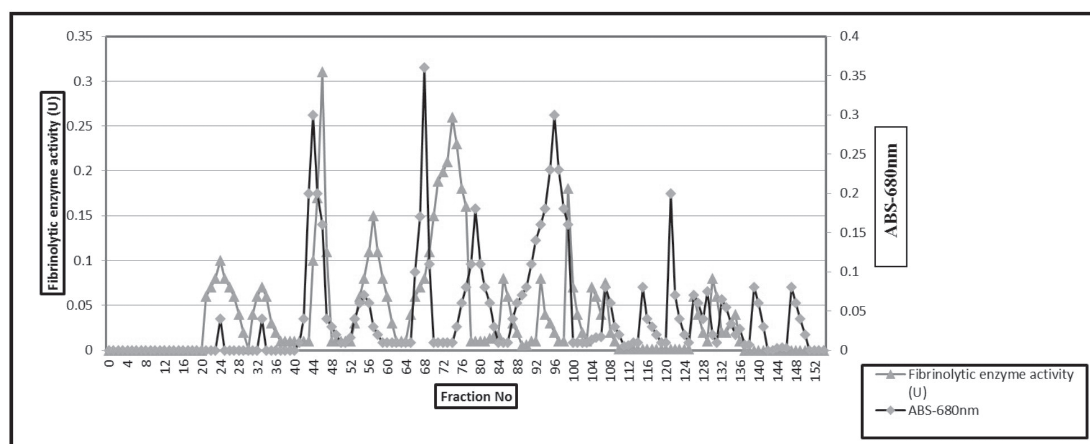


Fig 2. Anion exchange chromatography on DEAE Sephadex A-50

cm) equilibrated with 20 mM Tris-HCl buffer, pH 8. The absorbed protein solution was eluted at a flow rate of 1 ml/6 min with a discontinuous gradient ranging from 0.1 M to 1.0 M of NaCl dissolved in the same buffer. The elution profile of DEAE Sephadex A-50 chromatography is shown in (Fig: 2). From the elution profile, it was observed that the fibrinolytic enzyme was eluted as a well resolved single peak of fibrinolytic activity coinciding with a single protein peak at NaCl concentrations of 1.0 M. The fractions collected were determined for its total protein concentration and fibrinolytic activity. The active fractions 42 to 49 with high fibrinolytic activity were dialyzed and used for further studies. The summary of purification steps involved for fibrinolytic enzyme is presented in the Table: 1.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): The crude broth, precipitate obtained after ammonium sulphate precipitate and the purified fibrinolytic enzyme along with standard molecular weight markers were run on SDS-PAGE. Several bands were observed in the case of ammonium sulphate precipitate (Fig: 3), while purified enzyme showed a single band on SDS-PAGE, indicating a homogeneous preparation. The molecular weight of the fibrinolytic enzyme was determined by comparison of the migration distances of standard marker proteins (Lysozyme 14.4 kDa, Soybean Trypsin Inhibitor 21.5 kDa, Carbonic

anhydrase 31.0 kDa, Ovalbumin 45.0 kDa, Bovine Serum Albumin 66.2 kDa and Phosphorylase b 97.4 kDa). Depending on the relative mobility, the molecular weight of the protein band was calculated to be around 31 kDa. Thus, it was concluded that our fibrinolytic enzyme has a molecular weight of 31 kDa.

***Compatibility with detergents*:** Besides pH, a good detergent protease expected to be stable in the presence of commercial detergents. The fibrinolytic enzyme from *Bacillus cereus* GD55 unfortunately not showed stability and compatibility in the presence of local laundry detergents (Nirma, Henko, Surf excel, Wheel and Ariel). Our enzyme showed stability and compatibility in the presence of Rin. As such, the compatibility of our enzyme was studied with Rin in the presence of a 10 mM $CaCl_2$ for different time periods (5-60 min) at 37°C.The results are presented in Table: 2.

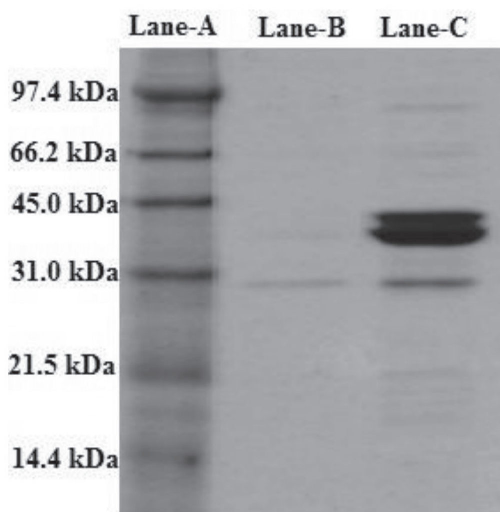
***Fibrinolytic potential of the enzyme*:** The blood stain was removed from a white cotton cloth by incubating the cloth in purified fibrinolytic enzyme for different time intervals. It was seen that enzyme produced by *Bacillus cereus* GD55 had a high capability of removing the blood stain (50 min), which indicates its potential in detergent industries (Fig: 4). In the present study the removal of blood stain by *Bacillus cereus* GD55 producing enzyme is a promising additive in detergent industry.

Conclusion

In the present study we report the production and purification of a new alkaline fibrinolytic enzyme from *Bacillus cereus* GD55, which may be a good candidate in pharmaceutical industry. Based on the above results and discussion the following conclusions are made. The analysis of SDS-PAGE indicates the molecular weight of alkaline fibrinolytic enzyme is 31 kDa, depending on the relative mobility. The purified enzyme specific activity was an increase of 11.59 fold than crude enzyme extract. Thus apart from proteases being used as detergent additives in detergent, for bio treatment of leather, as a thrombolytic agent

Table 2. Compatibility of fibrinolytic enzyme activity with commercial detergents in the presence of $CaC1_2$

Relative residual fibrinolytic enzyme activity (%)							
Time (min)	**Control**	**Ariel**	**Henko**	**Nirma**	**Rin**	**Surf Excel**	**Wheel**
0	100	100	100	100	100	100	100
5	0	0	0	0	10	0	0
10	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0
40	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0
55	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0



Lane A: Molecular mass markers; Lane B: Purified enzyme; Lane C: Crude enzyme

Fig 3. SDS-PAGE of alkaline fibrinolytic enzyme from Bacillus cereus GD55

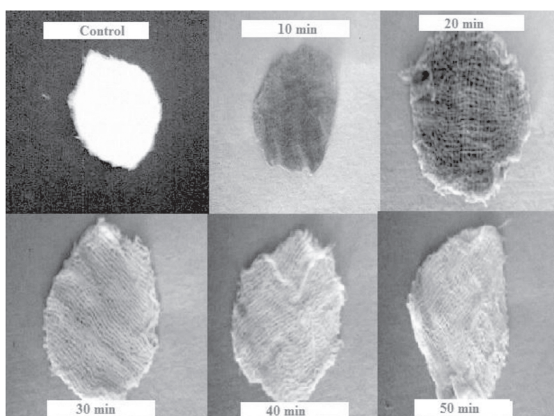


Fig 4. Removal of blood stains by alkaline fibrinolytic enzyme produced by Bacillus cereus GD55 at various time intervals

having fibrinolytic activity and being used for killing bacteria, viruses, mold and fungi, etc. This study clearly indicates that they can be effectively employed to remove blood stains and thus can be of great use and significance in medical industry. Based on our research findings, we are suggesting that the alkaline fibrinolytic enzyme produced by solid state fermentation from *Bacillus cereus GD55* can be useful to meet the industrial requirements as well as increasing demand of the global enzyme market. The success in achieving high yields of enzyme by employing SSF technology encourages to explore the possibility of SSF being an alternative process for the production of detergent additive alkaline fibrinolytic enzyme.

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Antibacterial activity of *Emblica officinalis* (Gaertn.) Fruits and *Vitex negundo* (L.) Leaves

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Abstract

Emblica officinalis (Gaertn.) and *Vitex negundo* (L.) are traditionally used medicinal plants found in tropical and subtropical regions of India. Antibacterial activity of different extracts of *V. negundo* leaves and *E. officinalis* fruits were evaluated by agar well diffusion method against different pathogenic bacteria viz., *Escherichia coli, Serratia marcescens, Pseudomonas aeruginosa* and *Bacillus cereus* in the present study. Maximum antibacterial activity was obtained against *E. coli* (ZOI = 17.0±1.0 mm and AI = 0.939) by methanol extract followed by aqueous extract (ZOI = 14.5±0.5 and AI = 0.801) of *E. officinalis* fruits. In case of *V. negundo* maximum zone of Inhibition was observed against *E. coli* by methanol extract (ZOI = 13.1±0.76 and AI = 0.723) followed by inhibition of *Serratia marcescens* (ZOI =13.1±0.76 and AI = 0.684) by the same extract. The MIC values of different extracts used in study ranged between 6.25-100 mg/ml. In this study, 6.25 mg/ml was recorded as MIC of methanol extract of *E. officinalis* fruits against *E. coli*. The results obtained from qualitative phytochemical analysis revealed that the plants contained bioactive secondary metabolites which may be connected with antimicrobial properties of plants. Therefore, these results clearly support the traditional use of *E. officinalis* fruits and *V. negundo* as a broad-spectrum anti-microbial agent against a wide range of microbes.

Keywords: Antibacterial activity, Phytochemical analysis, Medicinal plants, *E. officinalis, V. negundo*

Introduction

Normally, millions of bacteria exist in the human body as a normal flora. The pathogenic bacteria are recognized as foreign particles once they entered in the human body and are either killed or inhibited from multiplying profusely as immune response. In some cases, even a healthy system cannot prevent pathogenic bacteria from reproducing and spreading to cause bacterial disease as they disrupt the defense mechanism of the body (1).

In recent era, development of multidrug resistance in pathogenic microbes throws a challenge to scientists to find out the source of alternative medicine (2). Nature has been a source of a variety of medicinal remedies since human existence on earth and an equally impressive number of modern drugs have been isolated from natural sources, mostly based on their uses in traditional medicine. Many medicinal plants have been used for years to treat disease all over the world. Plants are rich source of different types of medicines because they produce a diverse range of bioactive molecules.

Higher plants, as a source of medicinal compounds, have continued to play a dominant and important role in the maintenance of human health since ancient times (3).

It has been estimated that 14-28% of higher plant species are used medicinally and that 74% of pharmacologically active plant derived components were discovered after follow up on ethno medicinal use of the plants (4). At present, nearly 30% or more of the pharmacological drugs are derived directly or indirectly from plants and their extracts dominating in traditional medicine systems and a common element in Ayurveda, Homeopathic and Naturopathic etc. (5). The effect of plant extract on bacteria has been studied by a large number of researchers worldwide. Much work has been done on ethno-medical plants in India. Among the 120 active compounds isolated from the higher plants and widely used in modern medicine today, 80 per cent show a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived (6). Herbalists tend to use extracts from parts of plants, such as the roots or leaves but do not isolate particular phytochemicals (7). The primary benefits of using plant-derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment (8). India has a rich flora that is widely distributed throughout the country. Herbal medicines have been the basis of treatment and cure for various diseases and physiological conditions in traditional methods practiced such as Ayurveda, Unani and Siddha. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases (9).

Emblica officinalis (Synonym *Phyllanthus emblica* Linn., commonly known as Amla or Indian gooseberry) is a small and medium sized deciduous tree found throughout India, Sri Lanka and Malaca, the fruits of which are highly used in traditional medicine (10,11). Dried fruits of amla are used in the treatment of haemorrhage, diarrhoea and dysentery in Unani system of medicine (12). The active ingredient that has significant pharmacological action in amla is "Phyllemblin". Various phytochemicals such as alkaloids, flavonoids, tannins and terpenoids are reported to have several pharmacological properties such as antimicrobial, antioxidant, antigenotoxic, and anti-carcinogenic effects. Mainly it is considered to be a safe herbal medicine without any side effects. Indian gooseberry is a traditionally and clinically proven fruit for both, its application and efficacy (13). The genus *Vitex* belongs to the family *Verbenaceae* and comprises of large shrubs or small trees. *Vitex negundo* Linn. is most commonly distributed in Gujarat is called Nagol in local language. It is a very common aromatic plant and is used in medicine. All parts of the *V. negundo* plant especially, its leaves contain numbers of secondary metabolites such as alkaloids, phenols, flavonoids, glycosidic iridoids, tannins and terpenes. Because of the richness in phytochemicals, leaf extract is employed as nervine tonic, tranquilizer and vermifuge (14).

Considering the vast potentiality of these two plants as sources for plant derived medicines with regard to antimicrobial drugs with reference to antibacterial agents, a systematic investigation was undertaken to screen the potential antimicrobial activity of fruits of *Emblica officinalis* (Gaertn.) and leaves of *Vitex negundo* (L.).

Materials and Methods

Collection of plant materials used in the study: Fresh fruits of *Emblica officinalis* (Gaertn.) and leaves of *Vitex negundo* (L.) were collected from the botanical garden of G. J. Patel Institute of Ayurvedic Studies and Research, New V.V.Nagar, Gujarat, India. The taxonomic identification of these plants was done by the taxonomist. Fresh plant materials were washed with water followed by air drying and then homogenized to fine powder and stored in airtight bottles at 4°C. Some information related to plants is given in table 1.

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Table 1. Therapeutic uses of medicinal plants used in the study

Scientific Name	Local Name	Family	Part Used in study	Therapeutic Uses
Emblica officinalis (Gaertn.)	Aamla	*Phyllanthaceae*	Fruits	Antipyretic, analgesic, antitussive, antioxidant, hepatoprotective, nephroprotective, cardioprotective, immunostimulant, antimicrobial, anticancer, anti-inflammatory, gastroprotective and dermoprotective (15)
Vitex negundo (L.)	Nagol	*Verbenaceae*	Leaves	Antihistamine, antioxidant, anticonvulsant, anti-inflammatory and antimicrobial, analgesic, antimalarial, insecticidal and pesticidal (16)

Preparation of plant extracts : The plant extracts were prepared by sequential cold maceration method using hexane, ethyl acetate, methanol and distilled water as a solvent (17). Fifty grams of dried powder of plant material was soaked in 250 ml hexane for 24 hr at room temperature under shaking condition at 120 rpm. This solution was filtered with the help of whatman No. 1 filter paper. The filtrate was collected in petri dishes and allowed solvent to be evaporated at room temperature. The dried extract was stored in 2 ml eppendorf tube and further used for antimicrobial assay after dilution. The filter cake was dried at room temperature and stored separately. The dried powder of filter cake was sequentially resuspended in 250 ml ethyl acetate, methanol and distilled water to prepare dried extract in each solvent. After extraction in each solvent, remaining filter cake was dried and further used with next solvent for extraction. All the dried extracts were stored at 4°C.

Sample Preparation: Test samples to perform antimicrobial assay were prepared by dissolving 100 mg and 50 mg of each extracts in 1 ml of dimethyl sulphoxide (DMSO) to prepare test samples of 100 mg/ml and 50 mg/ml concentration, respectively.

Microorganisms used in the present investigation: Microorganisms were procured from the Department of Microbiology, ARIBAS, New V.V.Nagar, India. Out of four bacteria used in the investigation, three Gram negative bacteria were *Escherichia coli* (MTCC No. 448), *Serratia marcescens* (local isolate) and *Pseudomonas aeruginosa* (MTCC No. 424) and a Gram positive bacterium was *Bacillus cereus* (MTCC No. 135). All the bacterial strains used in present investigation were maintained at 4°C on nutrient agar medium.

Preparation of bacterial suspension: Colonies of different strains of bacteria were aseptically transferred to the individual flasks containing fresh nutrient broth and further incubated at 37°C for 24 hr.

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Antibacterial assay using agar well diffusion method: The antibacterial activity of the test extracts was determined by agar well diffusion method (18, 19, 20). To perform antibacterial assay, initially the stock cultures of bacteria were revived by inoculating in broth media and incubated at 37°C for 24 hr. The agar plates of the Muller Hilton's Agar media were prepared. Each plate was inoculated with an aliquot (0.1 ml) of the bacterial suspension (10^5-10^6 CFU/ml) which was spread evenly on the plate under aseptic condition. Suspension in each plate was allowed to dry for 20-25 min. Then, wells having 6 mm diameter were made with the help of sterile cork borer in the solid medium and filled with test samples of 100 mg/ml or 50 mg/ml concentration. The positive and negative control wells were filled with gentamicin (Standard drug of 10 µg/ml conc.) and dimethyl sulfoxide (DMSO), respectively. All the plates were incubated at 37°C for 24 hr. The antibacterial activity of each extract was assessed by measuring the diameter of the zone of inhibition (in mm) around each well. Three replications were carried out for each extract against each of the test organism. Activity index for each extract was calculated by using following formula (21). Activity index (AI) = Inhibition zone of the sample/ Inhibition zone of the standard drug

Determination of relative percentage inhibition (RPI): The relative percentage inhibition of each test extract with respect to positive control was calculated by using the following formula (22).

$$RPI=\frac{100\ (X-Y)}{(Z-Y)}$$

Where, X= Total area of inhibition of the test extract; Y= Total area of inhibition of the solvent and Z= Total area of inhibition of the standard drug.

The total area of the inhibition was calculated by using following equation, area = πr^2; where, r = radius of zone of inhibition.

Determination of Minimum Inhibitory Concentration (MIC): Minimum inhibitory concentration (MIC) of different extracts with respect to different microorganisms was determined using broth dilution method (18, 21, 23). Mueller Hinton broth (HiMedia, Mumbai) was used for the antibacterial assay. All the extracts dissolved in DMSO were first diluted to highest concentration (100 mg/ml) to be tested and then serial two-fold dilution was done to form concentration range from 1.56 to 100 mg/ml of each extract. For broth dilution, 0.1 ml of standardized suspension of a bacterial strain (10^6 CFU/ml) separately was added to each tube containing various extracts at concentrations of 0 (control), 1.56, 3.12, 6.25, 12.50, 25.0, 50.0 and 100.0 mg/ml in broth medium. The tubes were incubated at 37°C for 24 hr for bacteria and observed for visible growth after mixing the tubes gently. The lowest concentration of the extract in tube that failed to show any visible growth was considered as the MIC of the particular extract against particular bacterial strain. A tube containing broth and inoculum but no extract was taken as control. Bacterial suspensions were used as negative control, while broth containing standard drug was used as positive control.

Qualitative phytochemicals analysis:The plant extracts were tested for the presence of different chemical constitutes like, alkaloids, tannins, saponins, cardiac glycosides, steroids, phenols and flavonoids according to standard protocols (24, 25).

Alkaloids (Mayer's test): The extract was evaporated to dryness and the residue was steam heated on a boiling water bath in presence of 1% HCl followed by cooling of mixture. The mixture was filtered and treated with six to eight drops of Mayer's reagent. The samples were then observed for the presence of turbidity or yellow precipitation which indicates presence of alkaloids in the sample. Tannins: To 0.5 ml of extract solution 1 ml of water was added followed by addition of 2-3 drops of ferric chloride solution. Blue-black colored precipitate was observed for

the presence of tannins. Saponins (frothing test): Five milliliters distilled water was added to 0.5 ml of extract solution. Frothing persistence indicated presence of saponins in the sample. Cardiac glycosides (Keller-Kiliani test): To the solution of 1 ml extract in 1 ml glacial acetic acid, few drops of $FeCl_3$ and concentrated H_2SO_4 were added. Green-blue color in the upper layer indicated the presence of cardiac glycosides. Steroids (Liebermann-Burchard reaction): Four milligrams of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated H_2SO_4 was added slowly and green bluish color was observed for the presence of steroids. Phenol (Folin's test): Two milliliters of Folin's reagent was added to 2 ml of extract. Solution was observed for appearance of brown color for the presence of phenol. Flavonoids: Four milliliters of extract solution was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5-6 drops of concentrated HCl was added and red color was observed for flavonoids for the presence of flavonoids.

Statistical analysis:Mean value and standard deviation were calculated for each test samples. Data were analyzed by Two-way ANOVA and p values were considered significant at $p > 0.005$.

Results and Discussion

Infectious diseases are the major cause of morbidity and mortality worldwide. The number of multidrug resistant microbial strains and the appearance of strains with reduced susceptibility to antibiotics are continuously increasing due to indiscriminate use of antibiotics and other antibacterial agents in treatment. The plant based traditional medicines were proven highly effective for their utilization as a source of antimicrobial compounds (26). The beneficial medicinal effects of plant materials typically result from the secondary metabolites present in the plant, although; it is usually not attributed to a single compound but a combination of the metabolites. Plants are potentially useful for the development of chemotherapeutic agents. The plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well being. The use of plant extracts with known antimicrobial properties can be of great significance for therapeutic treatment (27).

Antibacterial activity of different extracts of *V. negundo* leaves and *E. officinalis* fruits were evaluated by agar well diffusion method against different pathogenic bacteria (table 2 and table 3). In this study, highest antibacterial activity was observed against *E. coli* (ZOI = 17.0±1.0 mm and AI = 0.939) by methanol extract followed by inhibition of *E. coli* by aqueous extract (ZOI = 14.5±0.5 and AI = 0.801) of *E. officinalis* fruits. While, hexane extract slightly inhibited growth of *Serratia marcescens* and remaining bacteria showed resistance to the extract. Overall, it is clear that ethyl acetate extract having maximum antibacterial activity against all the test organisms, followed by aqueous and methanol extracts of *E. officinalis.* However, the extract having minimum antibacterial activity was hexane extract of *E. officinalis*, which could inhibit growth of *S. marcescens* only. In case of *V. negundo*, highest zone of Inhibition was observed against *E. coli* by methanol extract (ZOI = 13.1±0.76 and AI = 0.723) followed by inhibition of *Serratia marcescens* (ZOI =13.1±0.76 and AI = 0.684) by the same extract. Minimum antibacterial activity was observed of ethyl acetate extract of *V. negundo* against *S. marcescens*. On the other side, *Pseudomonas aeruginosa* showed resistance against all the test extracts of *V. negundo*. Activity index (AI) and relative percentage inhibition (RPI) is the estimate of effectiveness of the inhibitory action of the plant extract as compared to standard drug. Higher activity index (AI) and relative percentage inhibition (RPI) of the extract indicate higher antimicrobial activity of the particular extract against specific bacterial strain. As per results of AI and RPI, it is clear that *E. officinalis* fruits possess higher antibacterial activity as compared to *V. negundo* leaves with relation to particular test antibacterial drug used in the study. Thus,

Table 2. Antibacterial activity (zone of inhibition - ZOI) of different extracts of *E. officinalis* fruits and *V. negundo* leaves against tested pathogenic bacteria

Name of Plant	Extract	Concentration (mg/ml)	Zone of inhibition (mm) (mean ± SD)			
			Serratia marcescens	***Bacillus cereus***	***Pseudomonas aeruginosa***	***Escherichia coli***
Emblica officinalis	**H**	100	9.6±0.57	-	-	-
		50	8.5±0.5	-	-	-
	EA	100	13.3±0.5	13.6±0.57	13.0±1.0	12.3±0.57
		50	11.6±0.57	9.5±0.5	10.5±0.5	9.1±0.76
	M	100	10.6±0.57	10.0±1.0	8.8±0.76	17.0±1.0
		50	8.5±0.5	7.6±0.57	-	11.8±0.76
	DW	100	13.0±1.0	12.8±0.76	12.0±1.0	14.5±0.5
		50	9.6±0.57	9.6±1.15	9.3±0.57	11.6±0.57
Vitex negundo	**H**	100	-	10.6±0.57	-	8.1±0.76
		50	-	8.5±0.5	-	-
	EA	100	9.5±0.5	-	-	-
		50	-	-	-	-
	M	100	13.0±1.0	-	-	13.1±0.76
		50	10.5±0.5	-	-	9.5±0.5
	DW	100	8.6±0.57	9.6±0.57	-	11.3±0.57
		50	-	7.1±0.28	-	8.5±0.5
Gentamicin		10 μg/ml	19.0±1.0	15.1±0.76	15.0±1.0	18.1±1.04
DMSO		-	-	-	-	-

All values are mean ± SD, n=3 (p>0.005), H = Hexane extract, EA = Ethyl acetate extract, M = Methanol extract, DW = Distilled water (Aqueous) extract, (-) = No zone of inhibition, Zone of inhibition including 6 mm well diameter, DMSO = Dimethyl sulphoxide

extracts having higher AI and RPI, can be used to develop alternative drug from the plant as it is more effective on the pathogenic bacteria.

One of the study showed that the aqueous crude extract of *Emblica officinalis* possess strong inhibitory action against all the test human bacterial pathogens such as *Bacillus* sp., *Lactobacillus* sp., *Pseudomonas* sp., *Proteus* sp. and *Streptococcus* sp. by using agar well diffusion method (28). Varghese *et al.*, 2013 (29) screened the *in vitro* antibacterial activity of aqueous, ethanol and acetone extracts of fruits of *Emblica officinalis* against Gram-positive versus Gram-negative bacteria employing *Staphylococcus aureus* and *E. coli*, respectively. All the extracts exhibited significant antibacterial activity, more against *S. aureus* than *E. coli*. It is concluded from the previous study that *E. officinalis* is more inhibitory to gram-positive than the gram-negative bacteria. *Emblica officinalis* possess potent antibacterial activity against *Escherichia coli*, *K. ozaenae*, *K. pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa, S. paratyphi A, S. paratyphi B* and *S. marcescens* (30). It is proven that aqueous, ethanolic and acetone extracts of fruits of *E. officinalis* effectively inhibited the growth of the test bacteria

Table 3. Antibacterial activity (activity index and relative percentage inhibition) of different extracts of *E. officinalis* fruits and *V. negundo* leaves against tested pathogenic bacteria

Name of Plant	Extract	Concentration (mg/ml)	*Serratia marcescens*		*Bacillus cereus*		*Pseudomonas aeruginosa*		*Escherichia coli*	
			AI	RPI	AI	RPI	AI	RPI	AI	RPI
Emblica officinalis	H	100	0.505	25.53	-	-	-	-	-	-
		50	0.447	20.01	-	-	-	-	-	-
	EA	100	0.700	49.00	0.900	81.12	0.866	75.11	0.679	46.18
		50	0.610	37.27	0.629	39.58	0.700	49.00	0.502	25.28
	M	100	0.557	31.12	0.645	43.86	0.586	34.42	0.939	88.21
		50	0.447	20.01	0.503	25.33	-	-	0.651	42.50
	DW	100	0.684	46.81	0.847	71.86	0.800	64.00	0.801	64.18
		50	0.505	25.53	0.635	40.42	0.620	38.44	0.64	41.07
Vitex negundo	H	100	-	-	0.701	49.28	-	-	0.447	20.03
		50	-	-	0.562	31.69	-	-	-	-
	EA	100	0.500	25.00	-	-	-	-	-	-
		50	-	-	-	-	-	-	-	-
	M	100	0.684	46.81	-	-	-	-	0.723	52.38
		50	0.552	30.54	-	-	-	-	0.524	27.55
	DW	100	0.452	20.49	0.635	40.42	-	-	0.624	38.98
		50	-	-	0.470	22.11	-	-	0.469	22.05

H = Hexane extract, EA = Ethyl acetate extract, M = Methanol extract, DW = Distilled water (Aqueous) extract, (-) = No zone of inhibition, AI = Activity index, RPI = Relative percentage inhibition

Table 4. Minimum Inhibitory Concentration (MIC) of different extracts of *E. officinalis* fruits and *V. negundo* leaves against tested pathogenic bacteria

Test Organism	MIC (mg/ml)							
	Emblica officinalis				*Vitex negundo*			
	H	EA	M	DW	H	EA	M	DW
Serratia marcescens	25	12.5	25	12.5	NA	100	12.5	100
Bacillus cereus	NA	25	25	12.5	25	NA	NA	50
Pseudomonas aeruginosa	NA	25	100	25	NA	NA	NA	NA
Escherichia coli	NA	25	6.25	12.5	100	NA	25	25

MIC = Minimum Inhibitory Concentration (mg/ml), NA = Not assessed, H = Hexane extract, EA = Ethyl acetate extract, M = Methanol extract, DW = Distilled water (Aqueous) extract

including *E. coli*, *Salmonella typhi*, *Salmonella paratyphi*, *Bacillus* sp., *Proteus* sp., *Pseudomonas* sp. and *Klebsiella* sp., but maximally of *Staphylococcus aureus* (31). Some other workers have proved that *E. officinalis* can be used as a broad spectrum antimicrobial agent (32, 33, 34).

Different extracts of *Vitex negundo* leaves were investigated for its anti-microbial activity on five bacterial species viz., *Staphylococcus aureus, Proteus vulgaris, Bacillus subtilis, E. coli* and *Pseudomonas aerugenosa.* Among all extracts water:ethanol (50:50) extract showed maximum anti-microbial against all tested species (35) which supports present study that methanol and aqueous extracts have more antimicrobial activity. In other study, the antimicrobial activity of the leaves and bark of *Vitex negundo* L. was evaluated against three Gram-positive bacteria viz. *Staphylococcus epidermidis*, *Bacillus subtilis*, *Staphylococcus aureus* and five Gram-negative bacteria viz. *Escherchia coli*, *Salmonella typhimurium, Pseudomonas aeruginosa*, *Vibrio cholera* and *Vibrio alginolyteus*. Results from disc diffusion and agar cup methods showed promising antibacterial activity of all the extracts of both leaf and bark against *E. coli*, followed by *S. aureus* (36). One of the previous study indicated that, *S. mutase* was least sensitive bacteria towards methanolic extract of *Vitex negundo* whereas; *P. aerogenosa* was proven to be most sensitive among the test bacteria (37). Broad spectrum antimicrobial activity of *V. negundo* was proven by some researchers. (38, 39, 40, 41)

MIC values were evaluated for all the extracts which were showing antimicrobial activity against bacteria in agar well diffusion assay (table 4). The MIC values of different extracts used in study ranged between 6.25-100 mg/ml. In this study, 6.25 mg/ml was recorded as MIC of methanol extract of *E. officinalis* fruits against *E. coli*. In previous study, acetone extract maximally inhibited the growth of *S. aureus* and *E. coli* at MIC of the extract (0.1 μg and 1.0 μg, respectively). MIC for ethanol and aqueous extracts were 0.3 and 1.0 μg and 1.5 and 3.75 μg, respectively, for *S. aureus* and *E. coli* (29). It was found that MIC for methanol extract of *Vitex negundo* L. leaves was 12.5 mg/ml against *Serratia marcescens*. However in previous work, 2.5 mg/ml of methanol of *V. negundo* leaves extract was found to be MIC against all the Gram-positive and Gram-negative bacteria (36). Finding out MIC can be the key step towards development of formulations for the new antimicrobial drug against specific microorganism with efficient action.

Table 5. Qualitative analysis for phytochemicals present in different extracts of *E. officinalis* fruits and *V. negundo* leaves

Name of Phytochemical	*Emblica officinalis*				*Vitex negundo*			
	H	EA	M	DW	H	EA	M	DW
Alkaloids	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	-	+	+	+
Tannins	+	+	+	+	-	-	+	-
Sterols	-	+	+	+	-	-	-	+
Cardiac glycoside	-	-	+	-	-	-	+	+
Flavonoids	-	-	+	+	+	+	+	+
Phenol	+	+	+	+	+	+	+	+

H = Hexane extract, EA = Ethyl acetate extract, M = Methanol extract, DW = Distilled water (Aqueous) extract, (+) = Presence, (-) = Absence of phytochemical

Plant phytochemical constituents such as saponins, alkaloids, flavonoids, tannin and some other aromatic compounds are secondary metabolites those serve as defence mechanisms against many microorganisms, insects and other herbivores (42). In the qualitative analysis of phytochemicals, the methanol and aqueous extract of *E. officinalis* fruits showed the presence of alkaloids, tannins, saponins, cardiac glycosides, steroids, phenols, glycoside and flavonoids. While, methanol extract of *V. negundo* leaves indicated presence of all the phytochemicals analysed in present study, except sterol. Hexane extracts of both the plant showed presence of less phytochemicals as compared to other extracts which might be reason behind their reduced antimicrobial activity (table 5). However, results of the present study showed that ethyl acetate extracts of *E. officinalis* fruits lack in some phytochemicals as compared to aqueous and methanol extracts. But overall ethyl acetate extracts was proven to be most effective extracts against test bacteria which may be due to presence of more amount of phytochemicals with higher inhibitory activity. Therefore, extracts should be quantitatively analysed for all the phytochemicals which might lead to final estimate of phytochemicals responsible for the antibacterial activity. It has been suggested that the antimicrobial activity of the plant is mainly due to the presence of essential oils, flavonoids, terpenoids, alkaloids, tanins, saponins and other natural polyphenolic compounds or free hydroxyl groups in plant extracts (43). The results obtained from this study revealed that the plants contained bioactive compounds which may be connected with antimicrobial properties of plants. Presence of more bioactive phytochemicals in the extract may be the reason behind the higher inhibitory action of the particular extract (38). As results showed that methanol and aqueous have the presence of more phytochemicals, they have higher antimicrobial activity as compared to hexane or ethyl acetate extracts. However, phytochemicals presents in the extract might be dependent on many factors like, solvent used, method of extraction, plant material used, method of analysis used and many more. Panda *et al*., 2009 (36) evaluated phytochemicals such as alkaloid, flavonoid, carbohydrates, glycosides, proteins and amino acids, steroids,vitamin C, fat and fixed oil revealed presence of most of constitutes studied in polar extracts such as ethanol, methanol and aqueous extracts compared to non-polar extracts (petroleum ether and chloroform). Although we have worked for qualitative analysis for phytochemicals, further quantitative analysis of extracts for phytochemicals is required for the accurate measurement of the phytochemicals. The knowledge of extent and mode of action for antibacterial activity of specific compounds present in the plant extracts, may lead to the successful utilization of such natural compounds for treatment of bacterial infections by developing antibacterial drugs.

Conclusion

Emergence of multidrug resistance in microorganisms has increased the need of identification and validation of new classes of therapeutics from the inexhaustible source of medicinal plants. Antimicrobial properties of the metabolites derived from medicinal plants have recently gained interest of researchers as the herbal medications have always been the most reliable source of treatment to people. Antibacterial activity of different extracts of *V. negundo* leaves and *E. officinalis* fruits were evaluated by agar well diffusion method against different pathogenic bacteria. Methanol and aqueous extracts of *E. officinalis* fruits showed effective inhibitory action against all the test bacteria. While methanolic and aqueous extracts of *V. negundo* leaves inhibited all the pathogenic test bacteria except *P. aeruginosa.* Therefore, these results clearly support the traditional use of *E. officinalis* fruits and *V. negundo* leaves as a broad-spectrum anti-microbial agent against a wide range of microbes. Further study on identification and purification of the bioactive principle compounds from crude plant extract leads to effective practical applications in antimicrobial drug development.

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Evaluation of Phytoconstituents, Nutrient Composition and Antioxidant properties in *Moringa oleifera*- BhagyaKDM 01 variety

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Abstract

Moringa oleifera is a highly valued plant with a profile of important minerals and is a good source of protein, vitamins, β-carotene, amino acids and various phenolics.In the present study, methanolic extract of leaves and pods were analysed for phytoconstituents, antioxidant properties, nutrient, mineral and vitamin. The results of the antioxidant activities were moderate in comparison to the standard antioxidant and the leaf extract was superior tothe pod in terms of antioxidant potentials.The IC_{50}values for DPPH radical scavenging activity was 150, 240 and 14µg/ml for leaf, pod and standard respectively. Pod was a good source of carbohydrate, lipid, protein and amino acids as indicated by the results. The moisture, ash and crude lipid of leaves were 80.02%, 6.85% and 1.83% respectively; those of the pod were 74.06%, 7.18% and 2.32% respectively. The mineral composition unravels a high concentration of iron and calcium followed by sodium, potassium and magnesium. The present results revealed that, the leaves and pod contain an appreciable amount of nutrients and can be included indiets to supplement our daily nutrient requirements.

Key Words: *Moringa oleifera*- Bhagya KDM 01, antioxidant, DPPH, phytoconstituents, IC_{50}

Introduction

Moringa oleifera is the most widely cultivated species of a monogeneric family, the Moringaceae, which is native to the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan. It is also known as the horseradish tree, drumstick tree, benzolive tree, kelor, marango, mlonge, moonga, mulangay, nebeday,saijhan, sajna or Ben oil tree. It is a perennial softwood tree and for centuries has been advocated for traditional medicinal and industrial uses. All parts of the *Moringa* tree are edible and have long been consumed by humans (1). The uses for *Moringa*is in plenty as described by Fuglie (2): alley cropping (biomass production), animal forage (leaves and treated seed-cake), biogas (from leaves), domestic cleaning agent (crushed leaves), blue dye (wood), fencing (living trees), fertilizer (seed-cake), foliar nutrient (juice expressed from the leaves), green manure (from leaves), gum (from tree trunks), honey- and sugar cane juice-clarifier (powdered seeds), honey (flower nectar), medicine (all plant parts), ornamental plantings, bio pesticide (soil incorporation of leaves to prevent seedling damping off), pulp (wood), rope (bark), tannin for tanning hides (bark and gum), water purification (powdered seeds). *Moringa* seed oil (yield 30-40% by weight), also known as Ben oil, is a sweet non-sticking, non-drying oil

that resists rancidity. The plant has been advocated as an outstanding indigenous source of highly digestible protein, Ca, Fe, Vitamin C and carotenoids suitable for utilization in many of the so-called "developing" regions of the world where undernourishment is a major concern (1).

Recently, the pharmaceutical industries are facing many challenges favoring the use of plant natural products over the current chemo-clinical drugs available for the treatment of different diseases [3].Developing cheaper, effective, new plant-based drugs with better bioactive potential and the fewest possible side effectsis needed. Hence, attention has been directed toward biologically active extracts and compounds from plant species to fight against microbial diseases (4-9), as well as against degenerative diseases caused by free radicals (10).

A comparative study on antioxidant potentials in sprouts vs. seeds revealed that sprouts have higher antioxidant capacity compared to seed extracts (11). Plant derived drug serve as a prototype to develop more effective and less toxic medicines (4).

In the present study we reported phytoconstituents, nutrient composition and antioxidant properties in leaves and pods of *Moringaoleifera*- Bhagya KDM 01 variety.

Material and Methods

Collection and Extraction of plant material: The plant material was collected from breeders plot, washed and rinsed with water to remove all the dirt and unwanted particles and then macerated into small particles. For phytochemical screening and antioxidant studies, they were weighed and mixed with methanol and incubated for a week time. After one-week incubation the mixture was filtered using Whatman No. 1 filter paper and evaporated at room temperature. The dry material left off after evaporation was used for further studies.For nutrient analysis, extraction was followed with requisite modifications. The samples were dried at room temperature to remove residual moisture,then placed in paper envelope and oven-dried at 55°C for 24 hours (12). The dried samples were ground into powderusing pestle and mortar and sieved through 20-meshsieve. The resulting powder was used for the nutrients analysis.

Preliminary phytochemical screening: The methanol extract of *M. oleifera* was screened for the presence of various phytoconstituents viz. steroids, alkaloids, glycosides, flavonoids, carbohydrates, amino acids, prote ins and phenolic compounds as described by Kokate *et al*. (13).

Antioxidant activity

Total phenolic content estimation: The total phenolic content was estimated according to the method of Makkar et.al 1997 (14). The aliquots of the extracts were made up to the volume of 1ml with distilled water. Then 0.5ml of Folin-Ciocalteu reagent and 2.5ml of sodium carbonate solution (20%) were added. After mixing, solution was incubated at 90^0C for one minute and the absorbance was recorded at 725nm against the reagent blank. Catechol is used to prepare the standard curve and total phenolic content of the extracts were expressed as catechol equivalent in µg/mg of extract.

Total antioxidant capacity: The total antioxidant capacity was measured by spectrophotometeric method as described by Prieto *et al*. (15). A concentration ranging from 50- 250 μg^{-ml} of plant extract was added in an eppendorf tube with 1ml of reagent solution (0.6M H_2SO_4, 28mM sodium phosphate, 4mM ammonium molybdate mixture). The tubes were incubated for 90min at 95ºC. The mixture was cooled to room temperature and the absorbance was read at 695nm against blank. Ascorbic acid equivalents were calculated using standard graph of ascorbic acid. The experiment was conducted in triplicates and values are expressed as equivalents of ascorbic acid in μg^{-mg} of extract.

DPPH radical scavenging assay: The free radical scavenging activity of the extracts, based on the scavenging activity of the stable DPPH free radical, was determined by the method

described by Wong*et al.*[16]. Different concentrations ranging from 50- 250µg^{-ml} of plant extract was added to 4 ml of a 0.004% methanol solution of DPPH. After 30minutes of incubation at room temperature, the absorbance was recorded at 517nm. A control reading was obtained using methanol instead of the extract. Ascorbic acid was used as the standard controland the percentage inhibition was calculated from the optical density of the treated and control samples using the following formula. The inhibition curves were prepared and IC_{50} values were obtained.

% of inhibition = (A0 - A1) / A0 X 100

Where, A_0 is the absorbance of the control (without test samples) and A_1 is the absorbance of test samples.

Reducing power assay: The reducing power of the extracts was evaluated according to Oyaizu [17]. 1 ml solution of the extract (50-250 µg/ml) was mixed with equal volume of phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide and placed in water bath at 50ºC for 20 min. Then it was cooled rapidly and 1 ml of 10% trichloroacetic acid was added and vortexed, centrifuged at 800g for 10 min and its 1.5 ml supernatant wasmixed with equal volume of distilled water and 1 ml of 0.1% ferric chloride and left for 10 minutes incubation and absorbance was read at 700 nm. The reducing property of test sample was standardized against quercetin.

***Nutrient analysis*:**The moisture, fiber, ash and crude fats, of the samples were determined by proximate analyses.Moisture was determined by oven dehydration method at 105^{0}C up to the constant weight. Crude fat was determined by ether extraction method using soxhlet apparatus. Crude fiber was determined by acid digestion and alkali digestion method. Ash content was determined in muffle furnace at 550^{0}C for 6h. The protein was determined by Lowery's method and total sugarwas estimated by anthrone method and total amino acid was determined as described by Sadhashivam Manikham (18).

***Determination of Minerals and Vitamins*:** For minerals determination 0.5 g of each sample was wet digested with HNO_3: $HClO_4$ (2:1) for 2-3 h on heating mantle (19). Digested samples were filtered through 0.45 µm pore size Millipore filter and volume was made to 100 mL with distilled water. Concentration of Ca, Mg, Mn, Fe, and Zn, was determined onAtomic Absorption Spectrophotometer (Perkin Elmer AA Analyst 700) equipped with standard hallow cathode lamps as radiation source and air acetylene flames, while Na, Kand P concentration was determined on Flame Photometer. Vitamin C was determined in fresh samples by dichlorophenol Indophenol dye reduction method (20) and vitamin A and β carotene was determined as described by Harold *et al.* (21).

***Statistical analysis*:** All the experiments were carried out in triplicates. The result were pooled and expressed as mean ± standard error (SE). Base of the regression lines plotted for % inhibition versus concentration, inhibition concentration (IC 50) was calculated. The data were evaluated by one-way ANOVA and Microsoft office Excel 2007 software.

Results

Table 1. Results of qualitative phytochemical analysis of methanol leaves and pod extracts Antioxidant activity

Tests	Leaves	Pods
Carbohydrates	+	+
Proteins	+	+
Amino Acids	+	+
Steroids	+	-
Glycosides	-	-
Saponins	-	+
Alkaloids	-	+
Flavanoids	+	+
Tannins & Polyphenols	+	+

***Preliminary phytochemical screening*:** Phytochemicals are in the strictest sense of the word chemicals produced by plants (1). The phytochemical examination revealed the

presence of sterols, alkaloids, tannins, saponins, flavonoids, phenols etc. in the methanol extract of the leaf and pod of Moringathe results are tabulated in table 1.

Total phenolic content estimation: The total phenolic content of the three leaf extracts were compared with standard curve of catechol (y =0.005x-0.011, R^2=0.987) and the results were expressed as the number of equivalents of catechol (μg/mg of extract). Among the two methanolic extracts, leaf extract of *M.oleifera* showed prominent total phenolic activity (54μg of catechol/mg of extract) when compared to pod extract (27.93μg of catechol/mg of extract). The results were presented in Fig 1.

Total antioxidant capacity: Both the leaf and pod methanol extracts showed a very potent total antioxidant capacity. The phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as the number of equivalents of ascorbic acid (μg/ml of extract) (y=0.003 x+0.029, R^2=0.991). The ascorbic acid equivalence of methanolic extractswas shown in fig.2. Among the two extracts, total antioxidant capacity was found to be highest in leaves (55μg of ascorbic acid/mg of extract) followed by pod (22 μg of ascorbic acid/mg of extract).

DPPH radical scavenging assay: The comparison of the antioxidant property by DPPH

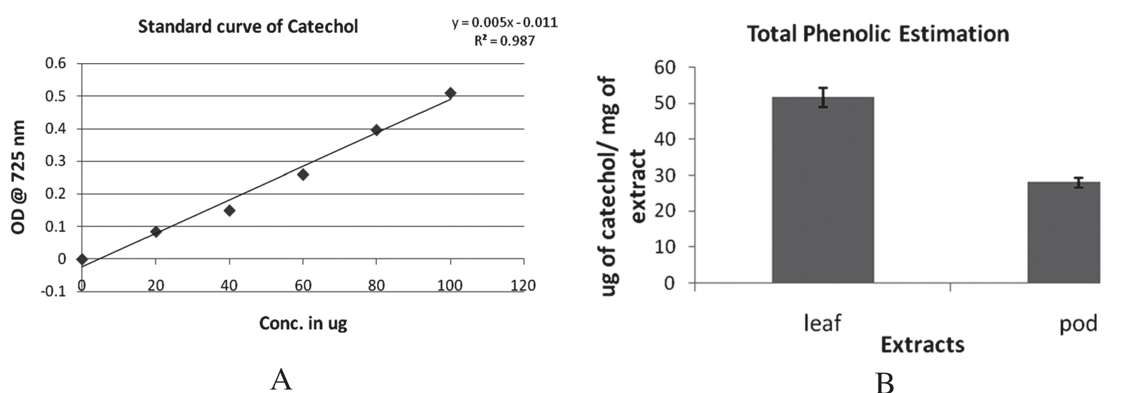

Fig. 1. Standard calibration curve of catechol for Total phenolic content estimation (A) and Catechol equivalence of methanol extracts of leaf and pod samples (B)

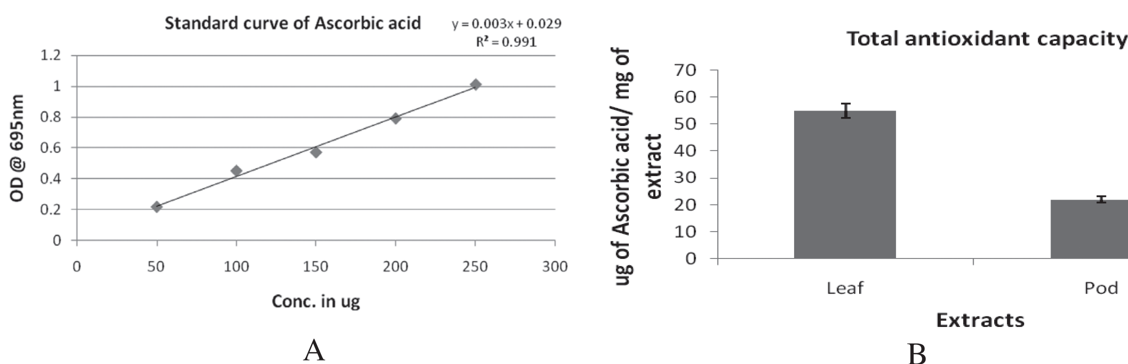

Fig. 2. Standard calibration curve of ascorbic acid for Total antioxidant assay (A) and its equivalence of methanol extract of leaves and Pods (B)

radical scavenging assay of the leaf and pod with that of the reference standard is depicted in Figure 3.The assay is based on the ability of an antioxidants present in the sample to decolorize DPPH free radical by virtue of their scavenging activities. The DPPH radical contains an odd electron that is responsible for the absorbance at 517 nm and also for the visible deep purple colour (19). The IC_{50} value revealed the more effectiveness of the methanolic leaf extract (150µg^{-mL})than the pod extract (240µg^{-mL}) comparable with the ascorbic acid (14µg^{-mL}). Both extracts exhibited a significant dose-dependent inhibition of DPPH activity.

Reducing power assay: The reducing power of a compound is related to its electron transfer ability and may serve as a significant indicator of its potential antioxidant activity [22]. The reducing power of the leaf and pod extract of *M. oleifera* increased in a dose dependant manner. The leaf extract was found to be remarkable than pod extract, which increased gradually with a rise in the concentration. The result of reducing power activity was shown in fig. 4.

Nutrient analysis: The nutrient profile of the leaves and pod of *Moringa* is given in Table 2.The

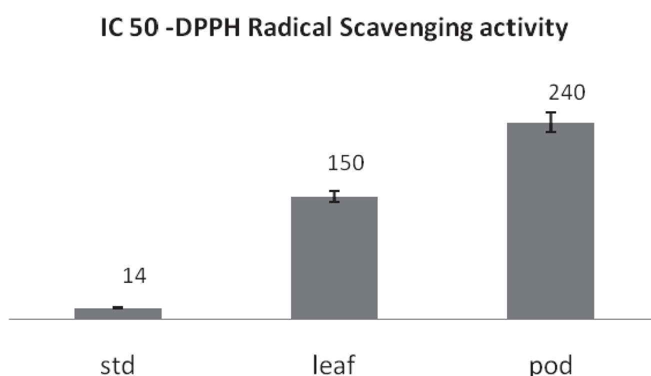

Fig. 3. IC_{50} values of DPPH radical scavenging activity of methanol extract of leaves and pods of *Moringaoleifera*

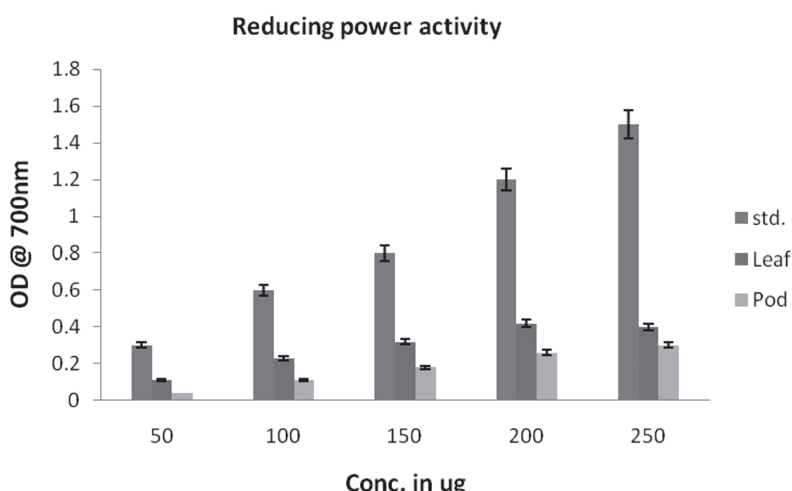

Fig. 4. Reducing power assay of methanol extract of leaves and pods

values for moisture content showed leaves having highest value (80.02%) than in the pod (74.06%).The ash content of leaves (6.85%) was lower than that of the pod (7.18%). The high ash content is a reflection of the mineral contents preserved in the food materials. The results therefore suggest a high deposit of mineral elements in the pod. Crude fat content of leaves (1.83%) were lower when compared to that of the pod (2.32%). The high concentration of fiber were accounted in pod (21.96%) rather in leaves (18.43%) and this makes it a more favorable vegetable since high fiber content of foods help in digestion and constipation. Nitrogen containing substances are found in fruits in different combinations: proteins, aminoacids, amides, amines, nitrates, etc. Among nitrogen-containing substances proteins and amino acids are most important (23). Pod had a higher protein (70 mg^{-g}) and amino acid (40 mg^{-g}) content than leaves (65 mg^{-g} and 30 mg^{-g} respectively) and this makes pod a good source of protein and amino acid when compared leaves. Carbohydrates are the source of energy required for various activities. In the present study the estimated sugar content in pod (130 mg^{-g}) stood to be higher than thatfor leaves (104 mg^{-g}).

Mineral and Vitamin analysis: The mineral profile estimated in the present study is shown in Table 2. The results unravel that leaf is rich source of all these minerals than the pod that promotes wellbeing in humans.Of the two samples leaves had a very high iron and calcium content with the pod sample having relatively lower. The high value of iron in these leaves makes them an additional source of iron. Calcium helps to build up strong bones and teeth so their consumption can add to daily calcium requirements of each individual. Sodium, potassiumand phosphorus are important for

Table 2. Nutrient profile of methanol extract of leaves and Pod

Sl no	Parameters	Leaves	Pods
01	Moisture (%)	80.02±0.01	74.06±0.01
02	Ash (%)	6.85±0.23	7.18±0.19
03	Crude fat (%)	1.83±0.17	2.32±0.16
04	Crude fiber (%)	18.43±0.28	21.96±0.23
05	Sugar (mg^{-g})	104.43±0.61	130.57±0.68
06	Protein (mg^{-g})	65.71±0.32	70.64±0.21
07	Total amino acid (mg^{-g})	30.42±0.12	40.54±0.16
08	β-Carotene(mg^{-g})	0.32±0.02	0.28±0.02
09	Calcium (mg^{-100g})	3.41±0.07	0.74±0.07
10	Potassium (mg^{-g})	2.21±0.03	3.28±0.01
11	Sodium (mg^{-g})	2.43±0.02	3.40±0.03
12	Phosphorous (mg^{-g})	3.21±0.04	2.16±0.03
13	Magnesium (mg^{-g})	0.50±0.01	0.49±0.01
14	Iron (μg^{-100g})	31.35±0.23	10.68±0.21
15	Copper (μg^{-100g})	7.30±0.08	5.73±0.07
16	Zinc (μg^{-100g})	21.24±0.37	18.53±0.41
17	Manganese (μg^{-100g})	56.72±0.31	48.52±0.27
18	Vit C Content (mg^{-g})	0.75±0.03	0.69±0.03
19	Vit A Content (IU^{-100g})	5.51±0.05	1.96±0.04

chemical reaction within the cells and regulate the transfer of nutrients to the cells. Sodium works in conjunction with potassium for extracellular fluid balances (24).Magnesium, manganese, copper and zinc was yet another mineral assessed (Table 4) which is important for any biochemical process in an organism, promotes balancing of minerals and it is necessary for normal job of muscles, and nervous (24) system, activity of hormones, manufacture of energy maintenance of health of reproductive system, immune system and regulation of an intimate rhythm and arterial pressure together with calcium (25).

Results of vitamins C, A and β-Caroteneare also shown in Table 4. Maximum concentration of these constituents was found in leaf than in pod implicating that leaves are good source than the pod. The concentration of Vitamin C, A and â-Carotene in leaf were 0.75mg^{-g}, 5.51IU^{-100g} and 0.32mg^{-g} respectively and in the pod their concentrations were 0.69mg^{-g}, 1.96IU^{-100g} and 0.28mg^{-g} respectively.Vitamin C (Ascorbic acid) is watersoluble vitamin required in high amount, as its loss is frequent from body. It participates in reversible oxidation-reduction system. Vitamin C prevents scurvy disease and also aids in the formation of folic acid derivatives, which are essential for DNA synthesis. Similarly, Vitamin A is necessary for a variety of functions such as vision, proper growth and differentiation, reproduction and maintenance of epithelial cells.

Discussion

Plants are very rich source of essential biochemical and nutrients such as carbohydrates,carotene, vitamins, calcium, iron, ascorbic acid, and palpable concentrations of trace minerals (26). A diet providing 1-2% of its caloric energy as fat is said to be sufficient to human beings, as excess fat consumption yields to certain cardiovascular disorders such as atherosclerosis, cancer and aging (27) and the fibre RDA nutritional purposes, considering to the amount andvalues for children, adults, pregnant and breast-feeding mothers are 19-25%, 21-38%, 28% and 29% respectively (28).The high value of carbohydrate and protein suggest its nutritional quality and this may be a veritable tool been used as source of body nourishment (29).

It is estimated that 70 biological trace elements are needed by all living things for the normal function of their metabolism, reproductive and immune system[30]. There has been a great emphasizing on the important roles of mineral and trace elements to human health and wellbeing. The presence of phytochemicals, in addition to vitamins and provitamins, have crucial nutritional importance in the prevention of chronic diseases, such as cancer, cardiovascular disease, and diabetes (31,32). Phytoconstituents like flavonoids, steroids and tannins are found to have analgesic and anti-inflammatory effects (33). Children, women of reproductive age and pregnant women are most vulnerable to micronutrient deficiency and anemia (34). Hence, they need food with high content of these minerals and vitamins.

Total antioxidant capacity by phosphomoly-bdenum method is based on the reduction of Mo VI to Mo V by the sample analyte and the subsequent formation of green P/Mo V complex at acidic pH. The phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as the number of equivalents of ascorbic acid (15). A comparable study on the antioxidant capacity by the phosphor molybdenum method was carried out by several researchers. [35-38].On the basis of results of three assays viz, DPPH, reducing power and total antioxidant capacity, the methanolic extract of leaf was found to be superior to the pod.

DPPH has long been recognized as a convenient reagent to quantify antioxidants in complex biological systems and has been widely used for this purpose.DPPH, a commercially available radical serves as the oxidizing radical to be reduced by the antioxidant and as the indicator for the reaction.The comparison of the results among the methanolic extract of leaf and pod have recorded moderate antioxidant

activities. Similar results were obtained by several investigators by using the DPPH assay to study antioxidant capacity of the medicinal plants for their relative antioxidant property. (39-42).

The reducing ability of a compound generally depends on the presence of reductants, which have been exhibited antioxidative potential by breaking the free radical chain (Fe^{3+} was transformed to Fe^{2+}), by donating a hydrogen atom. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reductive ability was measured in terms of Fe^{3+} to Fe^{2+} transformation in the presence of different concentrations of the methanolic extract of leaf and pod. Potent antioxidant capacity in terms of reducing capacity therefore can be attributed to reductones in the extracts because reducing properties are generally associated with the presence of reductones.The results were in conformity with observations of several investigations. (37, 43-45).

Conclusion

The leaf and pod methanol extracts of *M. oleifera*-Bhagya KDM 01 varietyshowed a moderate antioxidant activity in terms of DPPH, reducing power and total antioxidant capacity and yet these can be used as an easily accessible source of natural antioxidant however, the phytocons-tituents responsible for the antioxidant activityare not much clear. The nutritional analysisshown that pod is a good source of carbohydrate, lipid, protein and amino acids. Leaves are goodsourcesof minerals and vitamin A and C.Theresults suggest that the leaves and pod if consumedinsufficient amount could contribute greatly towardsmeetinghuman nutritional requirement for normalgrowthand adequate protection against diseasesarisingfrom malnutrition. From the result, the leaves and podsare recommend for continues use for nutritionalpurposes, considering to the amount anddiversityof nutrients they contains. The data confined in the present report is in the proximity with the earlier studies conducted in other varieties of *Moringa*. Chemical analysisalone-however, should not be the exclusive criteriaforjudging the nutritional significance of a plantparts.Thus,it becomes necessary to considerother aspects such as presence of antinutritional/toxicological factors and biological evaluation of nutrientcontent.

Competing interests: The authors have no conflict of interests to declare.

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Biochemical Studies During Sequential stages of Root and Shoot Differentiation in Callus cultures of *Cardiospermum halicacabum* L.

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Abstract

Present investigation was carried out on Cardiospermum halicacabum L. (Ballon Vine) - an important medicinal plant. Leaf explants were found to be the best for induction and growth of callus. Depending on fresh and dry weight of callus, high growth value callus was obtained on MS medium supplemented with 2, 4-D (1.0 mg/l) in combination with Kn (1.0 mg/l). This callus was selected for further studies of root and shoot organogenesis as well as biochemical studies. Regeneration of roots (15 days) from callus was observed on MS medium + BAP (0.5 mg/l) + NAA (0.3 mg/l) while shoots (16 days) on MS medium + BAP (0.5 mg/l) + NAA (0.1 mg/l). Starch content and reducing sugars were high in control or undifferentiated callus, which further increases significantly in root and shoot differentiating cultures. Contents of total soluble sugars, total soluble proteins and total phenols were lower in the control callus which increases in the root and shoot differentiating cultures. The activities of enzymes i.e. a-amylase, acid protease and peroxidase increased up to appearance of green patches (8th day) and were reached at peak on 12th day, the day that coincide with the visual appearance of roots and shoot. Conversely, the acid invertase activity decreases till the appearance of roots and shoots.

Key words: Ballon vine, callus, metabolites, enzymes, differentiation.

Abbreviations: MS, Murashige and Skoog; 2, 4-D, 2, 4-dichlorophenoxy acetic acid; IAA, indole acetic acid; IBA, á-indole 3-butyric acid; NAA, á-naphthalene acetic acid, BAP, 6-benzyl amino purine; Kn, kinetin.

Introduction

Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population and are important for socio-economic uplift of the human being. World Health Organization (WHO) has enlisted over 21,000 plants which have medicinal value. More than 2000 plant species are used in traditional medicines as evident from by Charak Samhita and Sushruta Samhita and 159 pharmaceutical companies and 3.5 billion people rely on these traditional medicines (5). *Cardiospermum halicacabum* L. (Ballon Vine) is an important medicinal plant belonging to family Sapindaceae. It is an annual or perennial climber. The medicinal properties of this plant are due to (b-sitosterol, D-glucoside, saponins and quebrachitol, stigmasterol, proanthocynidia and apigenin) present in the leaves. Knowledge on the control of differentiation has hardly grown since the demonstration that differentiation of organized structures in tissue culture in under the influence of growth regulators like cytokinins or a combination of cytokinin and auxin along with other components of the culture medium

(23). Although remarkable progress has been made in the area of gene transfer technology, little is known as to how plant cells differentiate in cultures or about molecular mechanism of in vitro differentiation (9). This unique property also offers an opportunity to investigate cellular and molecular basis of differentiation. Little is known about the intervening biochemical events occurring in the cultured cells undergoing organogenesis (plant regeneration), therefore elucidation of biochemical changes accompanying differentiation, will be decipher the underlying mechanism (22). There are only few reports on biochemical studies related to in vitro root organogenesis from callus in medicinal plants (8, 13, 16, 22, 25) in different plants. Therefore, analysis of various cellular metabolites and enzyme activities provide a reasonable and promising approach towards an understanding of the biochemical basis of the developmental pathway.

Materials and Methods

***Plant material and growth conditions*:** The plant material for this study was procured from mature plants grown in the Botanical Garden of the Department of Botany and Plant Physiology, CCS HAU, Hisar. The leaves obtained from field grown plants of C. halicacabum were surface sterilized with teepol solution for 10 min with vigorous stirring followed by washing in running tap water. These were then surface sterilized with 70% ethanol (1 min) followed by 0.1% mercuric chloride (3 min) and finally washed with sterile distilled water (4-5 times). The sterilized leaves were cut into small pieces (1 cm) and immediately inoculated, aseptically in the flasks containing MS (18) basal medium supplemented with 1.0 mg/l 2, 4-D and 1.0 mg/l Kn for callus initiation. The cultures were kept under photoperiod (2000 lux) of 16 h light at 26 ± 2°C. The high growth callus formed on 2, 4-D and Kn was sub-cultured on MS medium supplemented with BAP (0.5 mg/l) and NAA (0.3 mg/l) for root regeneration and MS medium supplemented with BAP (0.5 mg/l) and NAA (0.1 mg/l) for shoot regeneration. Various metabolites (starch, total soluble sugars, reducing sugars, total soluble proteins and total phenols) and enzymes (á-amylase, acid invertase, acid protease and peroxidase) activities were assayed during the sequential stages of roots and shoots differentiation from callus in Cardiospermum halicacabum as following:

1. Control- undifferentiated callus before kept on differentiating medium.
2. Part of the same callus (high growth value) used for control was kept on root differentiation medium [MS medium + BAP (0.5 mg/l) + NAA (0.3 mg/l)] and samplings were done at 4th, 8th, 12th and 16th day intervals respectively.
3. Part of the same callus (high growth value) used for control was kept on shoot differentiation medium [MS medium + BAP (0.5 mg/l) + NAA (0.1 mg/l)] and samplings were done at 4th, 8th, 12th and 16th day intervals respectively

***Extraction of metabolites*:** Extraction of metabolites was done by the modified method of Barnett and Naylor (2). One hundred mg of dry callus was homogenized in 80% ethanol (v/v) and centrifuged for 10 min at 10,000 g. The extraction procedure was repeated three times with the residue. The supernatants were pooled and the final volume was made to 5 ml with ethanol and used for estimation of total soluble sugars, reducing sugars and total phenols by using methods of Yemm and Willis (26), Honda et al. (11) and Amorim et al. (1), respectively. The pellet was hydrolyzed with 4 ml of chilled 0.2 N $HClO_4$ at 4°C for 24 h. The hydrolysate was centrifuged at 5000 g for 15 min and the supernatant was used for starch estimation by the method of Hassid and Neufeld (10). Total soluble protein in the callus was extracted in 0.1 M Tris-HCl (pH 7.5) by using pre-chilled pestle and mortar. The homogenate obtained was centrifuged at 10,000 g for 15 min at 4°C and total soluble proteins in the supernatant were estimated using the method of Bradford (4).

Enzymes activity analysis : One gram of fresh callus tissue was hand homogenised in pre-chilled pestle and mortar using 0.1 M Tris-HCl (pH 7.5) buffer containing 0.25 mM EDTA, 2.5 mM Cystein-HCl and 0.1% PVP. The homogenate was centrifuged at 10,000 g for 15 min at 4°C. The supernatant was used for estimating the enzyme activity of various enzymes viz. á-amylase, acid-invertase, acid-protease and peroxidase using the methods followed by Shuster and Gifford (21), Summer (24), Beevers (3) and Seevers et al (20), respectively. Total soluble proteins in the enzyme extract were determined as per the method of Bradford (4).

***Statistical analysis*:** All experiments were repeated at least twice, using 8-10 replicates (flasks) each containing three explants. The data were analyzed statistically using completely randomized design and the significance was tested at 5% level of critical difference.

Results and Discussion

***Callus induction and root differentiation*:** Among different explants (leaf, node and internode) used for callus induction and callus growth, the leaf explants was found to be the best for induction and growth of callus. Callus induction was observed from the cut ends of the leaves on MS medium supplemented with various concentrations of 2, 4-D and Kn within 15 days of inoculation. But on the basis of fresh and dry weight of callus, MS medium supplemented with 1.0 mg/l 2, 4-D and 1.0 mg/l Kn (Fig. 1; Table 1) was found to be the best for

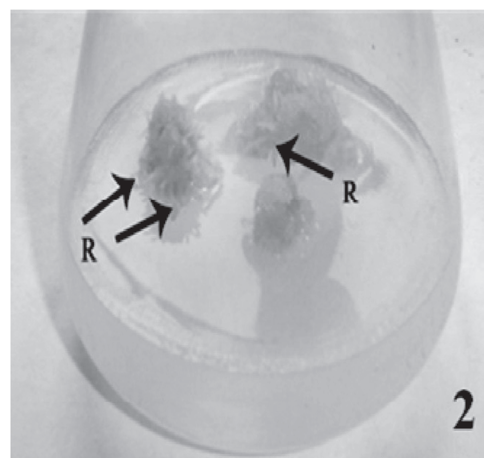

Fig. 2. Depicting formation of roots on MS medium supplemented with 0.5 mg/l BAP and 0.3 mg/l NAA from leaf derived callus in *C. halicacabum*.

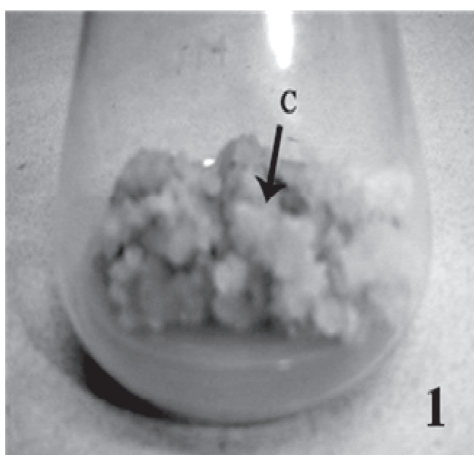

Fig. 1. Depicting high growth value callus (in terms of fresh and dry weight) from leaf explants on MS medium supplemented with 1.0 mg/l 2, 4-D and 1.0 mg/l Kn in *C. halicacabum*.

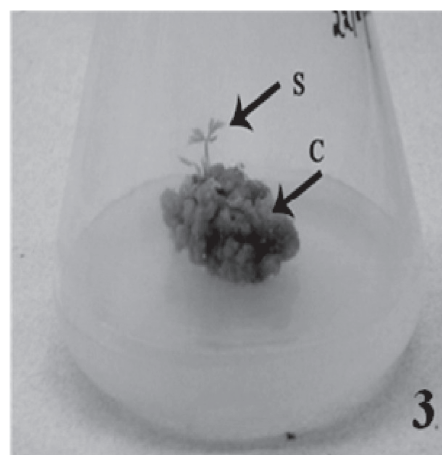

Fig. 3. Depicting formation of shoots on MS medium supplemented with 0.5 mg/l BAP and 0.1mg/l NAA from leaf derived callus in *C. halicacabum*.

good callus induction as well as growth with 100% callusing within 23 days of inoculation.

Any increase or decrease in concentration of both the growth regulator resulted in the reduction of callus induction and subsequent growth (Table 1). The explants therefore, require an optimum concentration of growth regulators for their differentiation into unorganized callus. In many other plants like *Medicago arborea* (16), Gloriosa (12), *Coffea arabica* (14), *Festuca arundinacea* (19) where 2, 4-D in combination with Kn was also used for callus induction. The 4 week-old yellowish white fragile callus sub-cultured on MS medium along with BAP (0.5 mg/l) + NAA (0.3 mg/l) showed visible roots formation (Figure 2) within 12-16 days and by decreasing the concentration of NAA i.e. 0.1 mg/l showed shoot formation (Fig.3; Table 2).

Table 1. Callus growth on various concentrations of 2, 4-D in combination with Kn supplemented to MS medium on fresh and dry weight of 4-weeks old calli derived from leaf explants of *C. halicacabum.*

Medium + Growth regulators (mg/l)	Weight of callus (g)	
	Fresh weight	Dry weight
MS + 2,4-D (0.5) + Kn (0.5)	2.239±0.059	0.255±0.019
MS + 2,4-D (0.5) + Kn (1.0)	2.117±0.107	0.200±0.019
MS + 2,4-D (1.0) + Kn (0.5)	2.164±0.082	0.215±0.032
MS + 2,4-D (1.0) + Kn (1.0)	**3.008±0.248**	**0.528±0.039**

Data presented is mean of three replicates

Table 2. Effect of growth regulators supplemented to MS medium on leaf callus [developed on MS + 2, 4-D (1.0 mg/l) + Kn (1.0 mg/l)] for root differentiation in *C. halicacabum.*

Medium + growth regulator (mg/l)	Visual growth of callus after 4-weeks *	Colour, Shape and Texture of callus	Differentiation (Days required)
Control	—	Brown, compact	-
BAP (0.5)			
+NAA (0.1)	+++	Green, globular, fragile	Shoots (16)
(0.3)	+++	Light brown, globular, fragile	Roots (15)
(1.0)	+++	Light green, globular, fragile	Somatic embryos (32)
BAP (1.0)			
+NAA (0.1)	+++	Yellowish green, fragile	Somatic embryos (36)
(0.5)	+++	Light green, fragile	Somatic embryos (30)
(1.0)	+	Green, smooth, compact	-
Kn (0.5)			
+ NAA (0.5)	+++	Creamish, globular, fragile	Roots (24) & Shoots (32)
(1.0)	+++	Green, globular, compact	-
Kn (1.0)			
+ 2,4-D (0.5)	++	Creamish, globular, compact	Somatic embryos (13)
(1.0)	+	Creamish, smooth, compact	Somatic embryos (13)

* — No callus, + Poor callus, ++ Moderate callus, +++ Good callus. Data presented is mean of three replicates

Cellular metabolites

***Root differentiation*:** starch content which was higher in control, increased from 0 to 8th day, after that the content decreased continuously till 16th day while total soluble sugars showed sharp increase up to 8th day then declined gradually up to root formation i.e. on 16th day (Fig. 4A). Prior to inoculation on rooting medium, the reducing sugars were more in the callus but a sharp decrease was observed during root formation. After the appearance of roots on 12th day, a little increase in the content of reducing sugars was observed in differentiating calli (Fig. 4A). The decrease in starch content could be due to decrease in the activity of synthesizing enzymes or increase in hydrolyzing enzymes. The decline in total soluble sugar content was associated with utilization of sugars for growth and differentiation process. Similar trend was reported in *Zamia furfuracea* (8) and *Chlorophytum borivilianum* (22).

Whereas, the total soluble proteins in root forming callus were increased up to 12th day as compared to control (Fig. 4B). Maximum protein content was found during roots initiation but after roots formation total soluble proteins start decreasing. During differentiation the cells are quantitatively changing their activities, new proteins have to be synthesized, thus protein content in callus forming roots is high. Similar observations were also reported by Mohapatra and Rath (17). The phenols were decreased during roots differentiation. On 12th day, roots differentiating calli had very less phenolic content as compared to undifferentiated callus (Fig. 4B). However, a little increase was observed on 16th day i.e. after roots formation. Phenols content decreased during differentiation could be due to that phenols participate in formation of cross-linking of cell wall constituents, which is catalyzed by peroxidase (15).

***Shoot differentiation*:** starch content showed a zig-zag trend i.e. increases from 0 to 4th day, then decreased on 8th and 12th day and then a little increase was found with the appearance of shoots on 16th day. Total soluble sugars showed sharp increase up to 4th day and declined gradually up to shoot formation i.e. on 16th day. A steady increase in reducing sugar content of calli was observed before shoot initiation i.e. upto 8th day but sugar content decreased during shoot formation (Fig. 5A).

Similarly, the total soluble proteins in shoot forming callus remained high throughout the

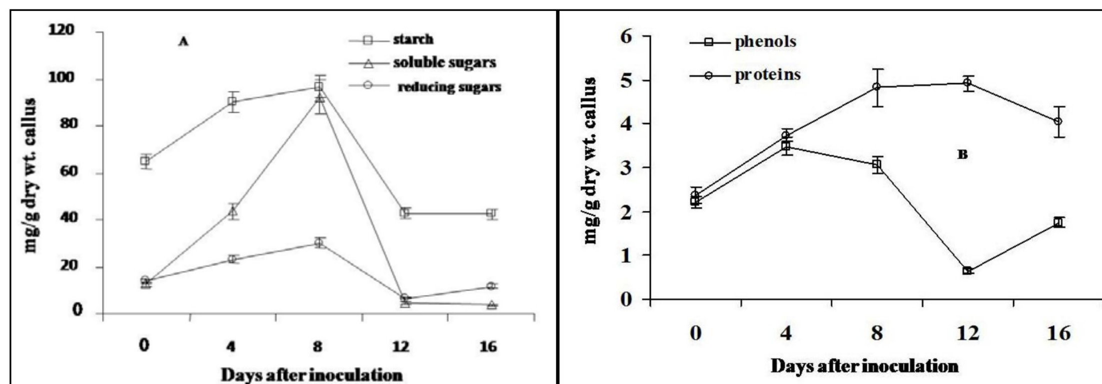

Fig. 4. Changes in the levels of starch, total soluble sugars and reducing sugars, A; total phenols and total soluble proteins, B; in *C. halicacabum* callus prior to inoculation on root forming medium (0 day) and after 4, 8, 12 and 16 day of inoculation (vertical lines indicate ± SE).

period of study as compared to control but the maximum content was found before green patches formation i.e. up to 8th day. The total phenolic also showed a zig-zag trend. However, shoot differentiation was accompanied by a drop in the phenolic content (Fig. 5B).

Enzyme activity

***Root differentiation*:** The a-amylase activity was low in undifferentiated calli. After the transfer of callus on rooting medium, the a-amylase activity increased up to 4th day and then a little decrease was observed up to 8th day (Fig. 6A). After that a marked increase was observed up to root formation. Whereas the undifferentiated callus had more acid invertase enzyme activity as compared to differentiated ones. The enzyme activity decreased up to 4th day from undifferentiated callus. After that the activity increased up to 12th day and then decreased up to 16th day i.e. root forming callus (Fig. 6A).

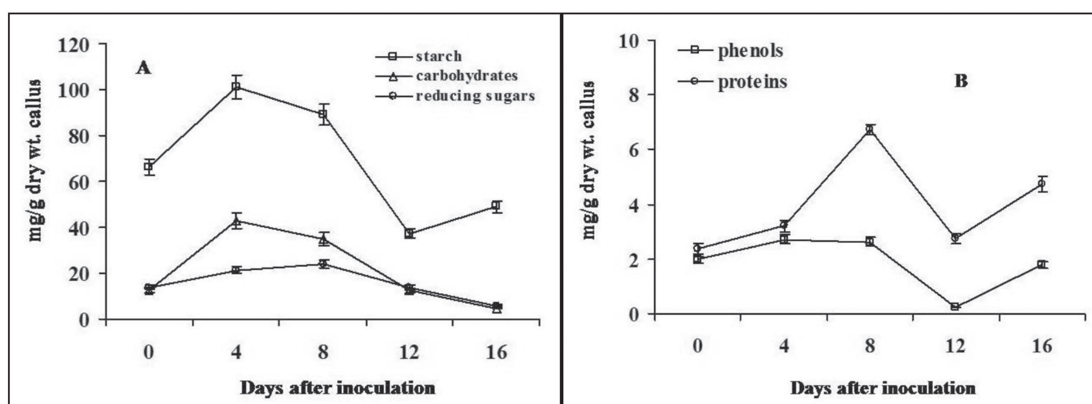

Fig. 5. Changes in the levels of starch, total soluble sugars and reducing sugars, A; total phenols and total soluble proteins, B; in *C. halicacabum* callus prior to inoculation on shoot forming medium (0 day) and after 4, 8, 12 and 16 day of inoculation (vertical lines indicate ± SE).

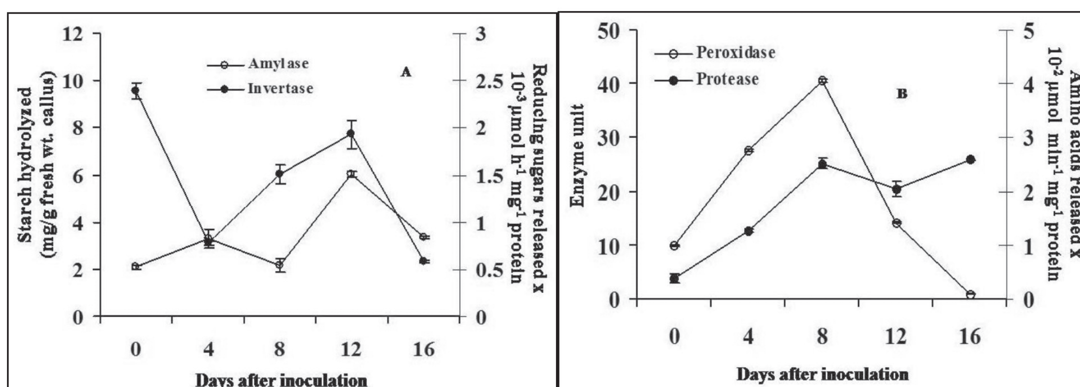

Fig. 6. Changes in the levels of á-amylase and acid invertase, A; acid peroxidase and acid protease B; enzymes activity in *C. halicacabum* callus prior to inoculation on root forming medium (0 day) and after 4, 8, 12 and 16 day of inoculation (vertical lines indicate ± SE).

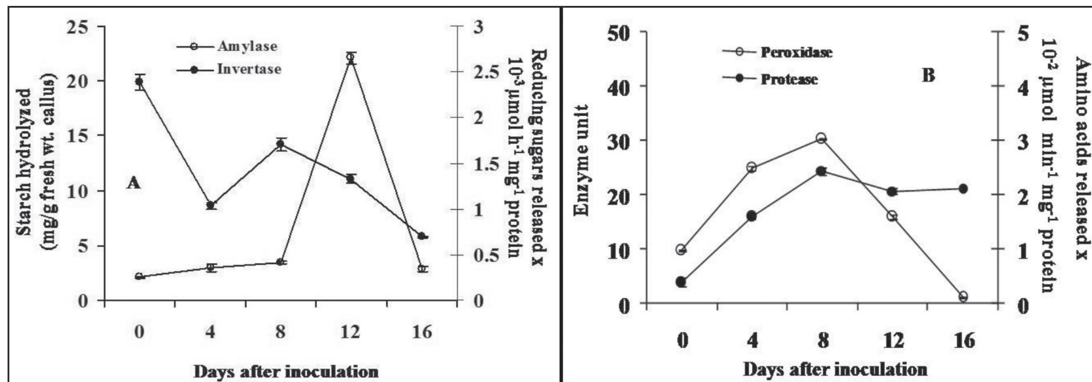

Fig. 7. Changes in the levels of á-amylase and acid invertase, A; acid peroxidase and acid protease B; enzymes activity in *C. halicacabum* callus prior to inoculation on shoot forming medium (0 day) and after 4, 8, 12 and 16 day of inoculation (vertical lines indicate ± SE).

The decrease in carbohydrate and the starch content in differentiating callus as compared to undifferentiated callus and higher á-amylase activity during differentiation seem due to the mobilization of carbohydrate reserves. Similar observation was reported by Singh et al. (22). Since the acid invertase is concerned with sucrose uptake and removal of sugars from the vacuole, the observed decrease in reducing sugars content and acid-invertase activity during differentiation may have a direct correlation with differentiation as suggested by Cuadrado et al (6). Acid-protease activity was least in control and it increased continuously up to 8th day after inoculation. Though a little decrease in content was noticed during root formation i.e. on 12th day was observed. Whereas the activity of acid-peroxidase was more in root differentiating callus than that of control callus. The peak activities of this enzyme were recorded on 8th day i.e. before roots formation (Fig. 6B). Higher proteolytic activity indicates high rates of degradation of pre-existing storage and other proteins required during differentiation as reported in *Cuminum cyminum* (7) and *Chlorophytum borivilianum* (22).

Shoot differentiation: The activity of á-amylase showed little increase up to 8th day, with sharp increase (more than 10 times) up to formation of green patches (12th day) followed by a sharp decrease in the activity on 16th day at shoots formation (Figure 7A). The acid invertase activity was more in callus prior to inoculation for differentiation and showed a continuous decrease in shoot differentiating calli (Fig. 7A) and showed a positive correlation with the reducing sugar content.

Acid protease activity was found to be higher under shoot forming conditions. Peak activity was observed on 8th day and was about 2 times higher than that in the undifferentiated callus (Fig. 7B). Total proteins were positively correlated with protease activity. The activity of peroxidase was more in callus than that of shoots differentiating calli. The peak activity of the enzyme was recorded on 8th day, associated with rapid differentiation (Fig. 7B).

Summary

The results from present study concluded that leaf explants gave best response for callus induction. Indirect regeneration was achieved through high growth value callus formed on MS medium supplemented with 2, 4-D (1.0 mg/l) + Kn (1.0 mg/l). MS medium supplemented with

BAP (0.5 mg/l) + NAA (0.1 mg/l) showed shoot regeneration while the increase in NAA concentration in the medium i.e. NAA (0.3 mg/l) showed root regeneration from the callus. Visible manifestation of cell differentiation includes greening of callus, variation in cell wall thickness but differentiation in such tissues involves differences in basic metabolic pathways i.e. utilization/formation of metabolites and changes in the enzyme activities. The results showed that decrease in metabolites like starch, reducing sugars, totals soluble sugar and total phenols while increase in total soluble proteins was found during root and shoot differentiation. Activities of enzymes viz. á-amylase acid invertase and peroxidase decreased while acid protease activity increased during root and shoot differentiation.

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Biochemical and environmental insights of declining vulture population in some Asian countries

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Abstract

Traces of diclofenac and its derivative compounds have been found in the carcasses of vultures across India and its neighboring countries, and it is known that the biomagnification of diclofenac from the consumption of infected domestic animal carcasses contributes to vulture mortality. However, reports also indicate that problems associated with their habits and habitats, food and feeding behavior, nesting and breeding, reproduction, epidemic and endemic diseases, and environmental factors, such as high temperatures and cyclones, might also be contributing factors. Adequate information is not available to confirm whether only diclofenac is the primary cause of vulture mortality versus their susceptibility to microbial pathogens, diseases or physiological conditions, such as oxidative stress due to diclofenac biomagnification. Death due to other contaminants or pollutants has also been not adequately studied. So future research may be able to determine whether the biomagnification of diclofenac and other organic/ inorganic pollutants or some other factors are responsible for vulture mortalities. Further investigations into the health issues related to life cycles and pathology need to be performed to restore the sharply declining vulture populations in India and across the globe. In this context, India is ahead of other countries in adopting recovery plans for vultures, for which the rate of decline of long-billed vultures is now 16% per year compared to that of oriental white-backed vultures which is 44% per year.

Key words: Diclofenac, biomagnification, remediation, eco-pharmacological effects, pollution, vulture extinction

1. Introduction

Vultures are highly significant in food webs as they play the key ecological role of consuming the carcasses of dead animals, which prevents the spread of diseases to livestock (1). Vulture populations at national and global scales are declining and are on the threshold of extinction. This has prompted researchers to restore their populations through laboratory and field interventions. An attempt is made in this article to identify the possible causes of and remedies for the decline of vulture populations with special consideration of the biomagnification of diclofenac from infected domestic animal carcasses. Emphasis is placed on the fact that diclofenac contamination cannot be the only cause behind declining vulture populations, and it is likely that several factors cause vulture mortality.

Different trophic levels stabilize ecosystems, and if one is lost, its entire ecosystem will be pushed into an unbalanced state. Threats to organisms at different trophic levels, such as producers, consumers or scavengers, eventually lead to disturbances in the food chain as well as imbalances in the food web (2). It is noteworthy that scavengers perform several crucial roles in a food chain without which the ecosystem's maintenance of dead carcasses will either stop or be delayed (3), which disturbs

the relationships between prey and predators and between producers and consumers (4, 5). Therefore, the absence of scavengers can lead to a serious crisis in an ecosystem (6), so the conservation of these species is especially important (7).

Many vultures such as *Gyps bengalensis* and *Gyps indicus* are now classified as critically endangered by the IUCN (12), and many vulture populations are either severely declining or already locally extinct, which is not only true for the Indian subcontinent but for many places across the globe (8-14, Fig. 1). Vultures usually do not kill animals but rather eat dead ones thereby keeping the environment clean (Fig. 2). Domestic animal carcasses are disposed of openly in the absence of safe alternatives, which not only leads to an increased risk of diseases, such as rabies, but also provides a platform for other livestock borne diseases, such as anthrax. Thus, it can be inferred that the scavenging role of vultures prevents the spread of dangerous diseases that could threaten wildlife, livestock and human beings (15). Therefore, it is clear that vultures are very important to terrestrial ecosystems (6). However, despite their role in maintaining the "balance of terrestrial ecosystems", a current topic of debate among ecologists and environmentalists, not much work has been performed to protect these species. For example, *Gyps* vulture populations across the Indian subcontinent began to decline in the 1990s and the process continues (15). Repeated demographic surveys have shown that the rate of decline was so rapid that elevated mortality of adult birds must be a key factor (16). Different nations have adopted several field and laboratory interventions and formulated different conservation strategies to protect vultures (17), but recovery efforts have not had the success anticipated, although the rate of population decline has slowed in countries such as India and Nepal (18). While India is ahead of other nations in the adoption of specific strategies for vulture conservation, the recovery rate has not reached the expected level.

Fig. 1. Vultures of India, reproduced after Taigor (15).

Fig. 2. Vultures feeding on carcasses in the Moyar Valley, Sathyamangalam Forest Division, Tamil Nadu (**A**) (after Ramakrishnani et al. (32) and in Nepal (B) (www.birdlife.org).

Multiple reasons have been ascribed to the mortality of vultures worldwide. In India and Nepal, the biomagnification of diclofenac from the carcasses of domestic animals to vultures is considered to be the main cause of vulture mortality (17-31). However, in the absence of specific studies or observations, this claim is difficult to accept (32). Other factors, such as problems with vulture habits and habitats, food, diseases, breeding, and environmental extremities may also contribute to mortality. These external and internal factors affect the normal physiology of animals and can lead to metabolic depression and eventually to death (33). One of the important cellular responses that creates metabolic depression in animals is oxidative stress (OS), which is resulted due to the oxidation of proteins, lipids and nucleic acids by the overproduced free oxygen radicals (34). OS is always positively correlated with the magnitude of any external and internal stress, so the susceptibility of vultures to OS due to infection by microbial pathogens, diseases or physiological disorders cannot be ignored.

2. The status of vulture populations in some Asian countries : In the protected areas of India, vultures numbers are few, i.e., from 13 to 65 individuals (15). Approximately four decades ago, two vulture species, namely, the Indian white-backed (*G. bengalensis*) and long-billed (*G. indicus*) vultures, were abundant (9, 15, 17), but, according to the reports of the Bombay Natural History Society (BNHS), these species are now on the verge of extinction (35). Beginning in 1985, Prakash et al. worked in Rajasthan's Keoladeo National Park, where white-backed vultures nested and long-billed vultures came to forage from nearby breeding sites. They observed the number of the park's white-backed go from a peak of 1,800 in 1985-86 to only 86 in 1998-99 while long-bills declined from 816 to 25 (36, Fig. 3). In 1999, data published from a BNHS study in Keoladeo National Park (KNP), a world heritage site, showed a 96% decline in the Indian white-backed vulture population and a 97% decline in the long-billed vulture population between 1985 and 1999 (35). Similar observations have been published elsewhere by different authors (8, 36, 37). In 2000, nationwide surveys revealed a similar decline in vulture populations throughout India (38). Later, a severe reduction in populations of *Gyps* species was observed by several ornithologists in neighboring countries, such as Nepal and Pakistan (16, 17, 27, 37, 39).

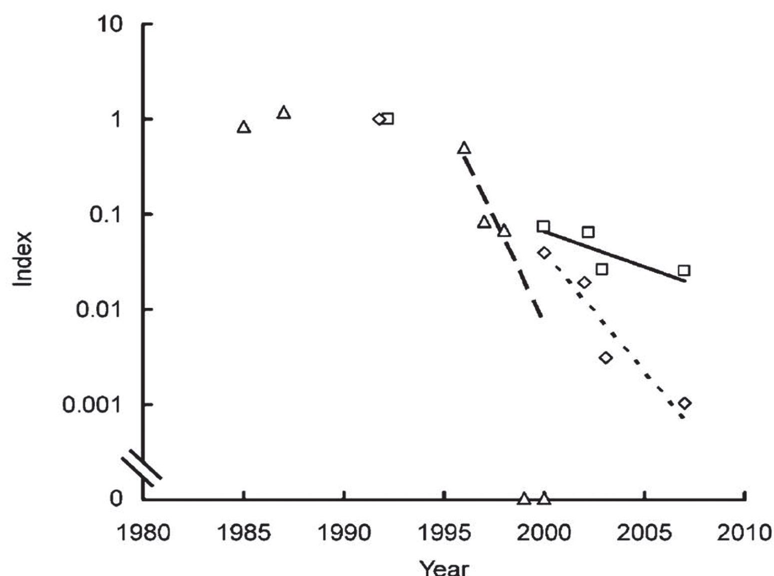

Fig. 3. Declining populations of *Gyps* vultures in India (reproduced from Pain *et al.* (17)). Points show population size index values on a logarithmic scale plotted against calendar year, which depicts the population as a proportion of its initial size. Triangles represent the number of active *Gyps bengalensis* nests in Keoladeo National Park, from Prakash et al. (36), expressed as a proportion of the average number in the 1980s. Indices of population size, relative to 1992, of *G. bengalensis* (diamonds) and *G. indicus* and *G. tenuirostris* combined (squares) in northern India were calculated from road transect count data as described by Prakash et al. (37). Lines depict fitted log-linear regression models (the dashed line represents 5 *G. bengalensis* at Keoladeo; the dotted line represents 5 *G. bengalensis* from road transects, and the solid line represents 5 *G. indicus/tenuirostris* from road transects).

3. Is diclofenac biomagnification the only cause of vulture mortality? : Multiple reasons have been assigned to the loss of scavengers, including vultures, in specific habitats (14, 40), and a number of studies have highlighted the persistent problems related to the causes and consequences of vulture extinction (17, 19, 35-37, 41-43, Fig. 2). Several reasons are believed to contribute to vulture mortality (Table 1). On the other hand, postmortem analyses of vulture carcasses from different locations have found traces of diclofenac and its derivative compounds in their tissues. While the biomagnification of a drug in an organism may not be the sole cause of mass mortality of its population, many authors have opined that diclofenac contamination, which contributes to renal failure and hepatotoxicity, is the only cause of vulture population decline in India (17-31). However, without properly designed experiments and data analysis, this claim may seem inconclusive. The available reports are inadequate for arguing whether diclofenac is the only main cause of vulture mortality or whether, following diclofenac biomagnification, the increased susceptibility of vultures to microbial pathogens, diseases or physiological disorders, such as OS followed by metabolic depression, are responsible for their mortality in large scale. The argument is that the detection of drugs such as diclofenac would be expected if an animal consumes diclofenac-contaminated food, including carcasses, more often. However, detecting diclofenac or its derivatives in the carcasses of animals cannot be the sole indication that diclofenac is the only cause of death. The discussions in this article

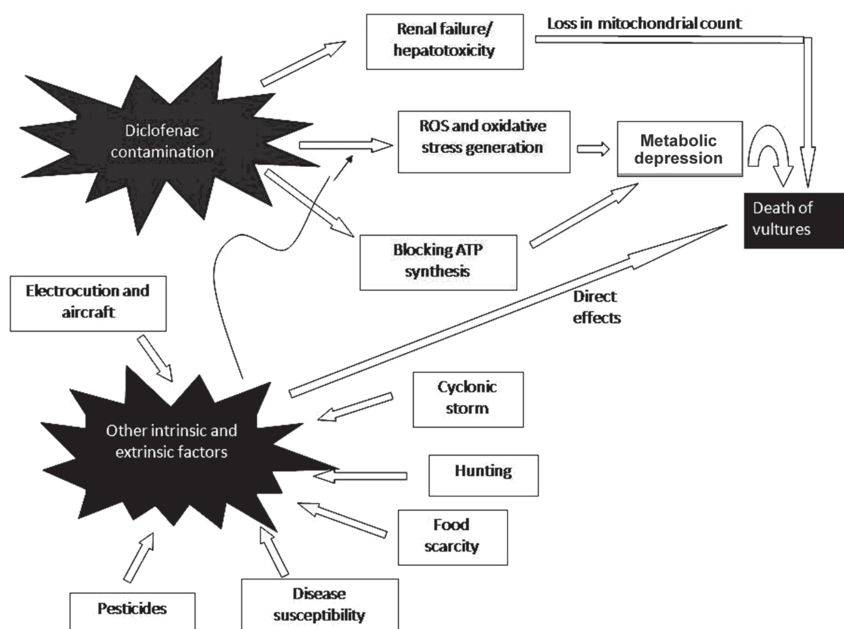

Fig. 4. Ray diagram showing the causes of declining vulture populations.

regard whether the biomagnification of diclofenac is the only cause of vulture mortality or if there are other factors based on the available literature. An attempt has been made here to note the possible role of elevated OS due to the consumption of diclofenac-contaminated carcasses in vulture mortality. An alternative viewpoint is given in an attempt to identify other possible causes that mediate and remedial measures that can arrest the rapid decline of vulture populations particularly in Indian subcontinent.

3.1. Global status of the research on diclofenac contamination and vulture mortality : A large number of pharmaceutical agents have been found to cause toxicity in several avian species, including vultures (23, 28, 29, 44). For example, the involvement of drugs such as carprofen, flunixin, phenylbutazone and ibuprofen in the deaths of vultures and other avian scavengers has been reported by several authors in India and other countries such as Spain and America (21, 28, 32, 45, 46, 47), and some articles also hint at the role of diclofenac contamination from domestic animal carcasses (17-31).

Diclofenac (2-[(2, 6-dichlorophenyl) amino] benzeneacetic acid, monopotassium salt) contamination from cattle carcasses provides a good example for discussion. It has been mostly studied in the context of vulture mortality by researchers in some Asian countries, such as India, Nepal and Pakistan. Diclofenac was first introduced in the UK in 1979 after being developed by Novartis (formerly named Ciba-Geigy) and was later used extensively in many countries (48). Diclofenac is used as a non-steroidal, anti-inflammatory and analgesic agent (25, 26), and it is not restricted to human beings but is widely used to treat domestic animals, especially cattle and goats (30, 31). According to Prakash et al. (43), if 10–20% of the estimated 503 millions of livestock in India die and become available for consumption by vultures annually (apart from the small portion eaten by people), 5-10% of these carcasses would have detectable

Table-1. Multiple reasons for the vulture mortality other than diclofenac contamination.

Reasons of Vulture mortality	***Place and year of Research***	***References***
Cyclone	Guntur and Prakasham area of Andhra Pradesh state, India, 1990	(60)
Mycoplasma infection	Changa Manga forest plantation area of Pakistan, 2004	(61)
Malaria	India, 2009	(36, 62)
Neck drooping	India, 2003	
Neck drooping and temperature		(65)
Hunting of vultures for meat by Bandola (Banda) people	Guntur and Prakasham area of Andhra Pradesh state, India, 1990	(60)
Consumption of infected carcasses by pesticides	Moyar Valley and different area in India, 2008	(25, 26, 32, 68, 69)
Metabolic depression related to oxidative stress and blocking in ATP synthesis	Across the world	(24, 51, 113)
Improper nesting and resting place	Turkey, 2008	(13)
Food scarcity and cannibalism	India, 1988, Keoladeo National Park, Bharatpur, Rajasthan, 2003	(41, 82)
Breeding related problems	India, 2003	(42, 87)
Pathological susceptibility	India and abroad, 2011	**(57, 69)**
Electrocution and aircraft	All part of the world	**(69, 90-92)**
Super cyclone	Coastal belt of the state Odisha, India, 1999	Personal observations of the authors

concentrations of diclofenac. However, given the short residence time of diclofenac in tissues, this would only be the case if all of the treated animals died within a week of receiving diclofenac. Taggart et al. (31) observed a 10% prevalence of diclofenac in samples from the carcasses of domesticated ungulates, which suggests that considerably more than 5 million courses of treatment are given annually and/or that the majority of animals being treated are fatally ill (44).

According to the American Society of Health-System Pharmacists, the overall bioaccumulation and retention rate of diclofenac is much higher than other anti-inflammatory and analgesic drugs and it is species specific (49). Therefore, bioaccumulation might be the main reason for the disruption of avian physiology in those animals that feed on the carcasses of domestic animals treated with diclofenac (24). Long-term administration of diclofenac to animals may result in severe pathological conditions, such as peptic ulceration, gastrointestinal bleeding, hepatotoxicity, renal papillary necrosis and renal failure, followed by death (19). If the above symptoms manifest themselves in vultures after they feed on carcasses contaminated with diclofenac, it can be inferred that diclofenac is the only cause of vulture mortality. However, the point remains as to whether this is the only major cause of the depletion of vulture populations from their habitats. It is true that diclofenac contamination has been detected in the carcasses of vultures, and many authors have strongly opined that diclofenac contributes to the catastrophic decline of vulture populations in

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many south Asian countries (21, 50). The major causes of death in vultures contaminated by diclofenac are renal failure and hepatotoxicity (24, 51, 52).

A total of 25 articles on diclofenac-related vulture deaths are currently available on the PubMed website (http://www.ncbi.nlm.nih.gov/pubmed?term=diclofenac%20vulture%20), but none of these articles aimed to discuss whether diclofenac contamination was either a "cause" or an "effect" or "both a cause and an effect" of vulture mortality. Additionally, none of the studies report whether vultures become susceptible to pathogens or other disorders after contamination or if death was mediated by diclofenac. The antimicrobial properties of diclofenac have been studied in microorganisms such as *Entamoeba coli* and *Mycobacterium tuberculosis* (53-55). Based on a preliminary investigation, Cunningham et al. (56, 57) claimed that an infectious disease with a viral etiology may be the cause of declining vulture populations. Although the authors could not find any pathogens in vulture carcasses, this does not necessarily mean that vultures are not susceptible to infectious diseases. Furthermore, the study was limited to particular areas, so it might be possible that diclofenac may impact the physiology of vultures both as an "effect" and also as a "cause" of susceptibility to other pathogens and disorders that can lead to death. On the other hand, Naidoo et al. (58) stated that, depending on its rate of elimination from the body, diclofenac is not likely to be a toxic agent in crow species but can be toxic to vulture species. Ramakrishnani et al. (32) also observed that diclofenac is not the only cause of vulture mortality. Based on such contradictory reports, further investigations are needed to find out whether multiple factors or diclofenac alone threaten vultures with large-scale destruction (10). Recently, Prakash et al. (18) reported that the rate of decline of *Gyps* vultures has slowed in India and Nepal due to the restricted use of diclofenac. In their 2011 survey, however, they observed that the populations of three species of vultures (*G. indicus, G. tenuirostris* and *G. bengalensis*) in both countries not only remained low but *G. bengalensis* showed a reverse trend. The reason for the slowing population decline was attributed to the ban on the use of diclofenac for veterinary purposes, but the authors did not consider other measures, such as captive breeding, the establishment of vulture restaurants and the rapid treatment of morbid birds, adopted by both government and NGOs to arrest the decline of vulture populations in these countries. Therefore, pointing to diclofenac contamination as the sole reason for the death of vultures seems unsupported. Research carried out by organizations and individuals using both laboratory and field methods has generated a list of possible causes other than diclofenac contamination for vulture mortality.

4. Other reasons for vulture mortality

4.1. Environmental factors : Biotic and abiotic environmental factors, such as *extreme* heat or cold, air and water pollution, cyclones, habitat loss, loss of forest canopy, imbalanced food chains, etc., are always detrimental to the survival of a species. For example, variable environmental drivers are found to push turkey vultures (*Cathartes aura*) for frequent movement and to change their habitat in North and South America (59). The magnitude of the adverse effects of such environmental factors is further enhanced by anthropogenic activities that threaten the lives of the species inhabiting an area. Although specific studies that attribute the mass destruction of vultures in India or elsewhere to such forces are lacking, environmental factors are viewed as playing a significant role in the decline of vulture populations. The author (BRP) has personally observed that no vultures were found along the coastal belt of Jagatsinghpur District in Odisha State, India, in the areas of Patrapada, Kakatpur, Astaranga, Kusupur, Nandhara, Olara, Padmapur, Paradeep, Belapur, etc. after the super cyclone with a cyclonic gale of 300 km /h which struck the area on the 29th and 30th October, 1999. At least 11 vulture colonies totaling more than 125 individuals

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disappeared from these areas after the storm. Similarly, in 1990, a severe cyclone reduced a local vulture population of approximately 100 to almost 0 in the Guntur and Prakasham areas of Andhra Pradesh, India (60). This implies that super cyclones might be a factor in the destruction of vultures by destroying the large trees used for nesting. Another cause of reduced vulture populations in the cyclone-affected areas could be the relocation of the birds prior to the arrival of the cyclonic storms, but no such documentation exists.

4.2. Pollution and other factors : Many vultures are colonial in nature (42), so it is possible that mass die-offs could occur after drinking water polluted with pathogenic organisms and organic or inorganic poisons. For example, the presence of a novel *Mycoplasma* species (*Mycoplasma vulturii*) in tissues of an oriental white-backed vulture (*G. bengalensis*) in the Changa Manga forest area of Pakistan (61) indicates that this *Mycoplasma* might be responsible for the decline of the vulture population. Although diclofenac was described as the major cause of death, it can be presumed that the presence of *Mycoplasma* species was responsible for the heterophilic inflammation in the trachea and bronchi of the bird, which could have caused its death (61). Although such studies have not been conducted, it could be helpful to identify both the primary (such as diclofenac) and secondary causes of death in vultures. However, the above study indicates that vultures can be affected by specific pathogens, and malaria pathogens can grow better in areas with polluted water. Although malaria is not a waterborne disease, due to the growth of the malaria vector *Anopheles* mosquito in waterlogged areas, the pathogens that cause malaria could be a cause of death in the wild population of the Indian white-backed vulture (62). Several organochlorine pesticide residues (p,p'-DDE, p,p'-DDT, HCH, dieldrin, etc.) have been detected in the tissues and eggs of *G. bengalensis* from different locations in India, such as Delhi and Mudumalai. Avian scavengers including vultures contaminated with organochlorine compounds also have been observed in Argentinean Patagonia of South America (63). Carneiro et al. (64) have detected heavy metal mainly Pb (24.15 to 25.98 µg/dl) in blood of Griffon vultures (*G. fulvus*) from Portugal and Catalonia, Spain. The authors propose that above concentration of Pb is sufficient to contribute for their impaired physiology especially in alleviating cellular redox regulatory capacity (64). The populations of Indian white-backed and long-billed vultures have been observed to exhibit a drooped neck posture followed by death due to pesticide contamination (36). Gilbert et al. (65) observed that neck-drooping is highly temperature dependent as the behavior serves thermoregulatory purposes, but the same group of authors has also suggested that neck drooping is not a purely temperature dependent behavior in adult vultures (65). Perhaps a combined analysis of neck-drooping as a function of temperature and pesticide contamination will clarify the above contradiction. It is assumed that pesticide contamination correlates with neck-drooping behavior in vultures, so neck drooping could be attributed to the drinking of contaminated water. In such cases, the death of vultures due to the biomagnification of diclofenac would be less probable. Therefore, it might be logical to say that water pollution might be a major reason for declining vulture populations, and future research may substantiate this claim. In the absence of specific experimental results, it may also be presumed that air pollution could drive vulture species to rapidly change habitats (59). As a consequence, adapting to new habitats with different ecosystem dynamics may be difficult; food scarcity would be the most important challenge. Improved techniques for spraying agricultural chemicals and pesticides and the pre-treatment of the gaseous pollutants from industry (especially from chimneys) and other activities (especially from vehicles) may diminish the threat of air pollution to arboreal animals such vultures.

Due to the arboreal and colonial nature of most vultures, environmental extremes, such as high rainfall, high or low temperatures and

cyclonic storms, may also affect their life span. Irregular and unexpected weather conditions have resulted from the loss of ecosystem balance in general and the loss of green forests in particular. The loss of green canopy always disturbs biogeochemical cycles, and disturbances to biogeochemical cycles, such as the water and carbon cycles, directly influence rainfall and temperature, respectively, while the nitrogen cycle influences canopy growth and development. All of the above processes influence avian life to a great extent (1, 66). As has already been mentioned, neck-drooping followed by mortality in oriental white-backed vultures (*G. bengalensis*) is correlated with a failure to thermoregulate under increased environmental temperature (65). On the other hand, some additional factors have also been found to be responsible for population decline. For example, after the decline in the vulture population due to a cyclone in 1990, the remaining vultures were further reduced by hunting (for meat) by the Bandola (Banda) people in the districts of Guntur and Prakasham of Andhra Pradesh (60). Similarly, one may repeat the personal observation by the author that the vulture population was almost absent from the coastal belt (especially in the coastal areas of Jagatsinghpur District and a few locations in Kendrapara and Puri Districts) of Odisha, India, after the super cyclone of October 1999. Such calamities cannot be avoided, but any further deterioration due to anthropogenic activities can surely be restricted.

4.3. Ingestion of contaminated food : The consumption of contaminated food can create multiple physiological disorders that can ultimately lead to death (24), and this may be the reason why Gilbert et al. (21, 65) suggested the establishment of vulture restaurants and monitoring with satellite transmitters. Such approaches could certainly reduce the risk of the consumption of contaminated food by the birds. Vultures feed in groups (67), which augments the probability of community destruction through feeding on contaminated carcasses. Therefore, dead animals contaminated with pathogens or poisons must be properly disposed of to avoid consumption by vultures. Many farmers spray cattle carcasses with pesticides, such as organochlorine and organophosphorous, to prevent the spread of foul odors (68), and these contaminated carcasses are eaten by vultures. Instances of hundreds of dead vultures due to the consumption of pesticide-contaminated carcasses are not difficult to find (69). It is reported that antimicrobial agents such as marbofloxacin in *Gyps fulvus* (70), penicillins and enrofloxacin in Egyptian vultures (71) can retain in their tissues. As a result this could lead to alter the balance in growth of the indigenous gut microflora, or the immunological status of these birds, making them more vulnerable to suffer infectious diseases and physiological depression. In this connection, geographical variation in cloacal microflora and bacterial antibiotic resistance in threatened vultures in relation to diet and livestock farming practices have been reported (71). It is clear from the above discussion that as with diclofenac contamination, other chemical factors could also be responsible for toxicity in vultures and other scavenger birds (32), and these factors need to be considered as priorities in vulture recovery plans (17, 72).

4.4. Food poisoning : Food poisoning may contribute to habitat-specific vulture mortality due to natural causes, such as toxic fungal or bacterial growth on carcasses or their improper disposal. Unfortunately, reports of vulture deaths due to fungal or bacterial infections caused by food are scarce. Although the occurrence of microorganisms, such as *Mycoplasma* (61), *Clostridium vulturis* (73) and several other microbiome (74) have been reported in oriental white backed vultures, cinereous vulture (*Aegypius monachus*) and new world vultures, respectively, and *Staphylococcus aureus* in Eurasian griffon vulture from Spain (75), investigations into microbial infections and their causative agents, such as food poisoning, should be included in vulture recovery plans.

4.5. Lack of proper nesting and resting places : Most vultures are arboreal and colonize large trees. Anthropogenic activities, such as encroachment on wild habitats and the cutting of large trees for various purposes, cause habitat destruction that displaces animals. Such phenomena have been reported for vultures (13). According to records available at the Regional Museum of Natural History in Bhubaneswar, India in 1992, slender-billed vultures (*G. tenuirostris)* were known to make their nests in large trees, such as banyan, mango, bullet wood etc., in the coastal belt of the Jagatsinghpur District of Odisha, but when the trees were cleared, the vulture population declined sharply.

4.6. Genotoxic factors : Genetic selection is the most important factor by which the fittest organisms survive to reproduce; this selection is automatic and includes changes at both the phenotypic and genotypic levels. Although molecular studies have been performed to determine the genetic evolution of vultures (76), research into the genotoxic factors responsible for declining vulture populations have received scant attention (77).

4.7. Nutritional problems : According to the report by the BNHS, nutritional factors are not a significant factor in vulture mortality. On the other hand, consumption of contaminated food may disturb digestion and its associated processes and thus result in the loss of nutrition and subsequent retarded growth and development. The problem could be severe in baby vultures as they consume a relatively high amount of food compared to adult birds. An audio-visual report has suggested that baby vultures are more susceptible to death related to food and nutrition (78).

4.8. Food availability : Carcasses are the only important source of food for vultures (1, 79), so the method of disposal of dead animals, such as releasing them into rivers, burying them in the soil and burning them in electric furnaces, can create food shortages. Therefore, if dead animals are disposed of in the open and away from human habitats, vultures might be able to consume the carcasses, which illustrates the concept of vulture restaurants. With a specific strategy and follow-up actions, the proper disposal of dead animals can mitigate negative consequences such as pollution and infection (80). Polis (81) claimed that most cases of cannibalism in nature occur due to food scarcity, and according to BNHS, although food scarcity may not be a cause of vulture mortality, the occurrence of cannibalism in vultures raises questions in this context (41, 82). Therefore, research is needed to ascertain whether food scarcity is a cause of vulture mortality, which can be addressed by studying the behavior of vultures during or prior to instances of cannibalism (83-85). Although vulture restaurants have already been established in a few locations in India, they can still be expanded into other parts of the country (21). They may serve as a beneficial and safe way to provide vultures with food and resting places, which may help them survive and reproduce to allow the rapid recovery of their populations.

4.9. Problems related to breeding : Declining populations can be revived by either slowing the death rate or raising natality (birth rate) along with increasing survivorship. If unnatural death is the reason behind the decline of a species then high natality may not be sufficient for species recovery. In this regard, little information is available on the problems faced by vultures during breeding and in sustaining their life, so identifying such factors may aid the recovery process through the adoption of artificial breeding techniques (72).

4.9.1. Induced breeding : Longevity and the breeding success of captive endangered birds are usually higher than in natural populations (86), so induced vulture breeding under conditions of captivity is therefore suggested if the natural natality is insufficient. Although induced breeding techniques are being applied in many countries, including India, the output is yet to increase remarkably. In India, a few captive care centers have been constructed, such as those at the Birshikargha Wildlife Sanctuary and

the Nandankanan Zoo. However, more such centers need to be set up in other parts of the country (42).

4.9.2. Incubation : Compared to adults, juvenile birds are usually more susceptible to mortality (78). Apart from induced breeding, the eggs of vultures may be collected from their natural habitat for incubation *in vitro*. Young vultures may then be released into the wild at a stage when their offensive and defensive abilities have developed and they can survive on their own. Instances of inceptive breeding failures in vultures suggest that proper care should be taken while breeding vultures in captive conditions (87).

4.10. Captivity plan for pathological susceptibility : Diseased or morbid vultures need to be identified and supervised to mitigate susceptibility to pathogens. It is believed that morbid birds exhibit signs of illness, including neck-drooping syndrome and inactivity, for nearly 30 days prior to death (36). Therefore, investigations at this stage would help in the assessment of the causes of morbidity and mortality of vultures. The analysis of infected birds may be helpful for drawing a clear picture of their patho-physiology, which has yet to be explored. The infected birds may be released following a complete recovery.

4.11. Epidemic and endemic diseases : Any endemic or epidemic disease must be investigated to take the appropriate remedial measures. Instances of vultures suffering from malaria have been observed (69), and Cunningham et al. (57) investigated 28 vulture carcasses, including adults and juveniles of both *G. bengalensis* and *G. indicus,* for any epidemic epidermal infections. These authors performed postmortem analysis of gut viscera, enteritis, vasculitis and gliosis, and although they did not identify the causative agents, the results of the pathological studies were mostly consistent with an avian viral etiology. It is important to note that Mallophaga species are known to attack bird populations and cause death (88, 89). Therefore, endemic or epidemic diseases could be the reasons for vulture mortality, so the patho-physiologies of vultures in different parts of the world require careful consideration.

4.12. Electrocution and air traffic : Electrocution as a probable cause of vulture death has been discussed by Gupta (69), but the author has maintained that this could not be a strong driver of extinction in avian populations, such as vultures. But a particular study indicated that a vulture without any known disorder died after coming into contact with high voltage electrical wires (69). The increased risk of electrocution in vultures has been attributed to their larger size and wing span, and the destruction of large trees leads them to use high voltage wires as perches. Some other species that are threatened by electrocution are the great Indian bustard, the sarus crane and some species of eagles. Nevertheless, more data are needed to support electrocution as a major cause of vulture mortality (90). However, the concern over collisions between aircraft and vultures, due to their large size, has already been discussed (91). In addition to electrocution, the large feathered forearms of vultures increase their overall risk from accidents, particularly with aircrafts (91, 92).

5. Reports by different organizations on vulture conservation : Out of nine species of vultures recorded in India, two species, namely, the Indian white-backed and long-billed, were the most abundant vultures in the Indian subcontinent approximately four decades ago. However, at present, they are on the verge of extinction and are classified as critically endangered by the IUCN (12). A report by the BNHS suggests that the decline in vulture populations cannot be due to factors such as food shortages, habitat loss, and toxicity, rather it could be due to a multifunctional disorder that needs to be properly studied. Over a decade ago, the scenario was reviewed by Indian and international experts at the "International Meeting on the Vulture Situation in India", which was held in New Delhi from 18th-20th September, 2000, and organized by the BNHS and supported by the

Royal Society for the Protection of Birds of the United Kingdom and the Ministry of Environment and Forests of the Government of India. The expert committee recommended the establishment of a nation-wide monitoring program to identify the reasons for the decline of vulture populations and suggest appropriate recovery plans. The establishment of a captive care facility for both sick as well as healthy vultures was another important issue raised by the committee. Accordingly, the Indian Vulture Disease Investigation Centre, the BNHS and the Poultry Diagnostic Research Centre (PDRC) of India, in collaboration with the Institute of Zoology of the Zoological Society of London, have been actively working on vulture diseases in India and abroad. The Darwin Vulture Project is one of the programs that established a dedicated vulture disease investigation center at the PDRC in Pune. On 11th June 2001, India's premier "Vulture Disease Investigative Centre" was opened to investigate the reasons behind declining vulture populations, and following that, a "Workshop on Monitoring Bird Populations" was organized in January, 2002, as part of the Darwin Vulture Project in collaboration with the Forest and Wildlife Department of Haryana. A total of 45 wildlife NGOs, wildlife departments from different states, the coordinators of the Indian Bird Conservation Network and research personnel studying vultures in India and neighboring countries participated in this workshop. They have developed a common protocol for monitoring vulture populations, including the identification and collection of *Gyps* species carcasses, postmortem examinations, etc. The details of the plans have been carefully explained, and they continue to be updated at www.vulturedeclines.org.

A training program is also conducted regularly for interested researchers in collaboration with the National Birds of Prey Centre of Newent, Gloucestershire, U.K. A "Vulture Care Center" in Panchkula District of Haryana was established at Birshikargha Wildlife Sanctuary in Pinjore. Internationally, two reputed organizations are working to conserve vulture populations. According to Pain et al. (17), the work of two research groups is especially important in this regard. The first group comprises the Forest Department of the state government of Haryana, the Zoological Society of London and the National Birds of Prey Trust, which later expanded to include a wide range of national and international organizations. The second group is composed of the Peregrine Fund, Washington State University and the Ornithological Society of Pakistan. While the first group, the BNHS consortium, is focused largely on India, the TPF/OSP group conducts complementary research programs in Pakistan and Nepal.

6. Modern research and vulture mortality : Under the present scenario of declining vulture populations, it may be difficult to obtain a clearance from the Department of Forests and Environment of the Government of India to sacrifice vultures for study. Furthermore, a comparative study of chemicals and other toxic materials, i.e., between diclofenac and agricultural organophosphorous compounds, which are consumed by Indian cattle while grazing, would be difficult to conduct. The majority of carcasses is usually destroyed, which limits further research. In this context, a report by Swan et al. (29) on Asian vultures demonstrates the recovery of their populations. In a comparative study of *G. africanus, G. bengalensis* and *G. indicus*, the authors observed that meloxicam, a veterinary medicine used in India, is of low toxicity to *Gyps* vultures and that its use in place of diclofenac would substantially reduce vulture mortality in the Indian subcontinent (29). Maryam et al. (93) recently discussed the use of different tissue culture techniques as a tool for the conservation of different endangered or threatened species. Tissue culture techniques are valuable because of their broad applications, and one of the most popular techniques is cryopreservation, which is the most effective technique for conserving the germplasm. Many endangered plants have been saved through this

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method of propagation, but there are some limitations as tissue culture methods can cause somaclonal variations. Apart from these limitations, tissue culture remains a major technique for saving commercially and medicinally important endangered species, but such techniques are widely used in the plant sciences but not in animal sciences due to various constraints (93). Therefore, if tissue culture techniques can be used on samples collected from morbid and healthy vultures, the factors responsible for their mortality may come to light.

Grivas et al. (78) described an automated surveillance system used to study siblicide in a bearded vulture (*Gypaetus barbatus*) nest in Crete from 2003–2006. The system is capable of operating autonomously for more than 1 week causing minimal disturbance to the birds. The system has two parts: 1) a nest-monitoring subsystem (camera, microphone, battery with a charge controller and transmitter with an antenna) supported by a solar panel and (2) a recording subsystem (antenna receiver, video signal controller and a PC remotely controlled through a GSM modem) that compresses the audio–video signal and provides real-time monitoring. Two-egg clutches with 7-day laying and hatching intervals were recorded while hatching asynchrony was determined through an analysis of the bioacoustic data. The food delivery rate and number of feedings to the first chick increased after its sibling hatched. Furthermore, it was observed that 98% of the aggressive interactions were initiated by the dominant chick and resulted in an average of 246 pecks per day. Supplementary feeding did not produce a significant correlation between feeding rate and sibling aggression, and the 2nd chick survived for 5 days before dying as a result of starvation accelerated by sibling aggression. On the basis of these results, the authors concluded that measures aimed at increasing the survival of the second chick should be undertaken when it is 1–2 days old. Although the above surveillance technique was used for monitoring the ethology between newborn and mother birds, it enables research on the morbidity of birds in nature, and it will undoubtedly help to reduce the steep decline of vulture populations in nature.

The analysis of metabolic indices is immensely important to the study of several core evolutionary concepts in animal biology, such as population ecology, life history tradeoffs, senescence, longevity and sexual selection in free-ranging organisms (94). Oxidative metabolism is one such metabolic pathway where O_2 performs a major role in directly or indirectly regulating the biochemical processes related to the oxidation of nutrients to produce energy. The physiology of OS comprises the respiration of oxygen (O_2) by mitochondria, the leaking of O_2 to produce reactive oxygen species (ROS), the oxidation of tissues by ROS, the response of both enzymatic and non-enzymatic redox regulatory molecules or antioxidants against the level of ROS produced and the generation of ATP molecules. The status of all of the above biomolecules has a direct or indirect relationship to health and longevity. Therefore, the analysis of OS indices and antioxidant defense parameters is also of immense importance to animal biology, such as in the study of disease susceptibility, organ failure, and longevity in free-ranging organisms (34, 94). Therefore, the role of OS in relation to both the external and internal factors responsible for vulture mortality may be extrapolated.

7. Role of oxidative stress and metabolic depression in vulture mortality : During the exposure of an animal to various insults in their natural habitat, the normal physiology of animals is compromised, and one of the important outcomes is the interruption of oxygen (O_2) consumption and the availability of O_2 to mitochondria under conditions of stress (95-97). Stress can limit or elevate the supply of O_2 to the mitochondria depending on an organism's metabolic status (97). As a result, it may concomitantly generate oxidants ROS under either a limited or elevated O_2 supply because both of these conditions are known to trigger OS

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pathways in animal tissues (34, 98, 99, 100). Reactive oxidants or ROS, such as the superoxide radical ($O_2^{•-}$), H_2O_2 and the hydroxyl radical ($^•OH$), are generated as byproducts of normal oxidative metabolism (101). During internal respiration, electrons are leaked from the mitochondrial matrix to the inter-membrane space through the complex I and III enzymes of the electron transport chain (ETC) (34, 95, 96). Therefore, ETC complex I and III enzymes act as the main hub for $O_2^{•-}$ generation (102-105). Under normal physiological conditions, approximately 1-5% of the O_2 consumed by mitochondria is incompletely reduced to $O_2^{•-}$ radicals, and this incomplete reduction of O_2 becomes > 5% under stress. In mitochondria, the 1st, 2nd and 3rd reductions of O_2 produce $O_2^{•-}$, H_2O_2 and the highly reactive $^•OH$ molecules, respectively (34, 106). All of the above molecules have high oxidant activity and, if not neutralized, can oxidize biological macromolecules including lipids, proteins and nucleic acids. $O_2^{•-}$ is catalyzed to H_2O_2 by superoxide dismutase (SOD) (107, 108), and H_2O_2 is neutralized to H_2O and O_2 by the catalase enzyme (CAT) (101, 109). H_2O_2 is also broken down by the glutathione peroxidase (GPx) enzyme in the presence of reduced glutathione (GSH), and oxidized glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (GR) with the help of NADPH. $^•OH$ and other ROS are neutralized by small molecular-weight, non-enzymatic antioxidants, such as ascorbic acid (AA), GSH, non-protein sulfhydryl (-SH), vit-E, vit-C, etc (110, 111). Under normal physiological conditions, a balance is maintained between ROS and antioxidants in the cells of aerobic organisms (95); the lower level of ROS, especially H_2O_2, can also be useful for animals due to its role in various signal transduction processes (112), but an insufficient level of antioxidants can result in greater ROS accumulation in cells. Due to an imbalance between O_2 availability and consumption by the animal and ultimately by the mitochondria, the activities of complex mitochondrial enzymes and antioxidants create favorable conditions for more ROS production and tissue oxidation. This results in increased lipid peroxidation (LPx), protein oxidation (carbonylation: PC), nucleic acid adduct formation and, finally, a disorder known as OS (34, 95, 96, 98, 111). It should be noted that the levels of all of the above biomolecules involved in OS physiology are altered as a function of any internal or external insult, such as environmental extremes, possible genotoxic stress, pollution, food scarcity, infection by contaminated food, food poisoning, nutritional problems, susceptibility to pathogens and stress due to epidemic and endemic diseases. Thus, there could be a correlation between the role of OS in the decline of vulture populations under the above conditions of stress.

In relation to the generation of ROS, birds have been found to be susceptible to OS after exposure to diclofenac (102). Considering the increase in the level of LPx and the enhanced activity of the two major redox regulating enzymes, SOD and CAT, with the decrease in the concentration of GSH in the liver, chicks have been observed to experience OS under a minimum diclofenac dose of 0.8 mg/kg body weight (51). The level of LPx as an index of OS has been found to increase in the liver of Japanese quail (*Coturnix coturnix japonica*) with the intramuscular administration of 5 mg/kg of diclofenac. This has been observed in a variety of birds, including vultures (24, 51, 52). Diclofenac (0.42 ìmol) has also been found to induce a substantial increase in the level of ROS in the cultured, isolated kidney cells of both the African white-backed vulture (*Gyps africanus*) and the domestic chicken (*Gallus domesticus*) (24). A recent review found that the acute hepatotoxic effects of diclofenac reduce/impair ATP synthesis (113); ATP synthesis is the final step in oxidative phosphorylation, and this pathway is highly linked to the generation of ROS and OS (98-111). Therefore, the role of cellular biochemical processes that have toxic end products, such as ROS in OS, may have exaggerated effects on nephrotoxicity and hepatotoxicity by diclofenac in vultures. Such

biochemical processes may amplify the toxic effects of diclofenac in birds in general and in vultures in particular. This prediction is supported by the observation that the susceptibility of vultures to renal tubular damage caused by diclofenac resulted from a combination of increased ROS production, interference with uric acid transport, and the duration of exposure (24). On the other hand, both OS and the blocking of ATP synthesis are responsible for the initiation of metabolic depression in animals which ends in cell death (102). So, in addition to renal failure and hepatic toxicity caused by diclofenac in vultures, ROS toxicity and OS-mediated metabolic depression seem to be coupled with the above physiological disorders. In combination or alone, all of the above factors could contribute to mortality.

The mechanism under which diclofenac can induce ROS followed by cell death in vultures could be explained through the loss of mitochondrial membrane fluidity in the kidney (102). *In vitro* experiments with the cultured kidney cells of vultures showed that diclofenac can induce cell death as evaluated by the inability of the cell culture to reduce the dye 3-(4, 5-dimethylthiazol-2-yl)2, 5-diphenyltetrazolium bromide. With the mitochondria being the only organelle capable of reducing the above dye to formazan, the resultant cell death could only be due to the death of the cellular mitochondria. This explanation is supported by the findings of Ng et al. (114, 115), especially with the toxicity of diclofenac being associated with a 200% increase in ROS production in vulture kidneys. Therefore, the hepatotoxic or nephrotoxic effects of diclofenac in vultures seem to be associated with the generation of ROS and the subsequent OS which could lead to mortality in birds.

8. Possible strategies to revive vulture populations : If a species is facing the possibility of extinction, then investigations into the health problems related to its life cycle and pathology must be conducted so that appropriate measures can be taken. India is ahead of other countries in taking steps to develop and implement recovery plans for vultures. As a result, the rate of population decline of the long-billed vulture, though rapid, is slowing substantially being 16% per year as opposed to the catastrophic decline of the oriental white-backed vulture at 44% per year (Fig. 3, 17). The following steps might be taken to recover their population.

Vigorous research into its biology, pathology and metabolism must be performed, and proper funding should be provided for research programs on their nesting ecology, roosting sites, breeding success and feeding ecology. Research laboratories in and around the vulture community must be established, which should also keep records on morbid vultures. General awareness programs should be put into practice to promote the proper disposal of dead animals while assessing their condition, especially as related to disease, while normal carcasses should be made available to vultures for consumption. The proper health and nutritional care of vultures both in captivity and in nature should be ensured through the establishment of vulture restaurants. Captive breeding and rearing should be encouraged and the identification of natural and artificial hazards, and remedial measures must be undertaken. Regular workshops and hands-on training programs (for carcass submission, preliminary analysis, treatment of ailing birds etc.) must be organized for researchers and other interested people laying emphasis on vulture surveillance and monitoring.

9. Concluding remarks : Based on the preceding discussion, it seems that there could be multiple issues behind declining vulture populations besides diclofenac contamination, so restricting the veterinary use of diclofenac may not be sufficient to restrict the decline. On the other hand, irrespective of the cause, morbid birds face a variety of physiological insults related to metabolic depression, such as the generation of ROS and OS, the loss of mitochondrial count, etc.

It is also clear that it is not too late to prepare an action plan to find the causes and

corresponding remedial measures to address the decline in vulture populations. Specific scientific and social strategies should be put forward to revive vulture populations, and there are instances such as the recovery of the rhinoceros population in India that suggest that the reversal of a declining trend of a particular population is possible. Programs such as the "Coastal Ocean Monitoring and Prediction System", the "Integrated Coastal Mapping and Management", and the "Bay of Bengal Program", which were instituted to protect coastal ecosystems in India, must be executed at the level of both government and organizations to protect declining vulture populations.

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Nitric Oxide (NO) and Hydrogen Sulfide (H_2S): Molecular Targets for Vascular Muscle Relaxation

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Abstract

Nitric oxide (NO) is produced in endothelial cells by nitric oxide synthase. This NO plays an important role in normal pathophysiology and homeostasis of human body. NO exerts a surfeit of biological effects in the cardiovascular system. Hydrogen sulphide (H_2S) is synthesized naturally in the body from L-cysteine mainly by the activity of two enzymes, cystathionine-γ-lyase and cystathionine-β-synthetase. The H_2S plays a vital role in vasorelaxation. Both NO and H_2S works by different mechanism to produce the effect of vascular muscle relaxation. The present review focuses on the molecular enzymatic targets for NO and H_2S for producing vascular muscle relaxation with recent advances and studies.

Key Words: Vasorelaxation, L-cysteine, cystathionine-γ-lyase, cystathionine-β-synthetase, (NO) nitric oxide and Hydrogen sulphide (H_2S).

Introduction

Small molecular weight gasses are amongst the most studied biological mediators over the past twenty years. Gaseous mediators such as nitric oxide (NO) and hydrogen sulfide (H_2S) are now accepted as a most important of such mediators in human body mediating blood flow, neurotransmission, immune reaction, mucosal integrity and intonation of inflammatory reactions. The most important physiological role of NO is in cardiovascular and inflammatory diseases. In cardiovascular system NO produces vasodilatation which is ligand mediated and flow dependent, inhibition of vasoconstriction by inhibition of angiotensin II and sympathetic vasoconstriction, anti-thrombotic by inhibition of platelet adhesion to the vascular endothelium, anti-inflammatory by inhibition of leukocyte to the vascular endothelium and anti-proliferative. NO is secreted as a pro-inflammatory mediator of arthritis in joints (1-7). In recent years, physiological roles of hydrogen sulfide (H_2S) have been documented, and there is promising evidence that this endogenous gaseous substance can modulate inflammatory processes. Indeed, H_2S donors have been exposed to reduce edema formation and leukocyte adherence to the vascular endothelium, and to inhibit pro-inflammatory cytokine synthesis. Moreover, an H_2S donor also increases resistance of the gastric mucosa to injury and speed up repair. H_2S probably works as vascular smooth muscle relaxant both in vitro and in vivo by opening vascular smooth muscle K^+_{ATP} channels (8-10). There is a need to design more NO and H_2S releasing drugs for the therapeutic application as vascular muscle relaxation potency which can be used in cardiovascular complications as well as pain management. The present review discusses the different molecular and enzymatic targets for designing new molecules for vascular muscle relaxation action.

***cGMP mediated Soluble Guanylyl Cyclase*:** With cGMP signalling being as crucial as it is to

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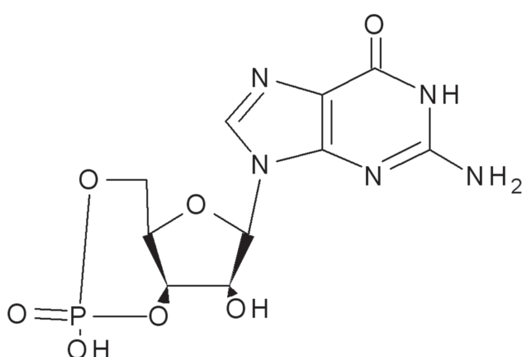
the physiologic functions of the heart and vasculature, in many cardiovascular diseases major factor is dysfunction at any level of the cGMP signalling.

Structure of guanosine 3', 5'-cyclic monophosphate : Because of endothelial cell dysfunctioning both systemic and pulmonary hypertension results; which further results in disorders like vascular smooth muscle dysfunction, systemic and pulmonary hypertensive and ischemic heart disease, cardiac myocyte dysfunction, hypertrophic and ischemic heart disease as well as cardiomyopathy and heart failure (Figure 2). Dysfunctional cGMP signaling has also been concerned in dysfunctional mitochondrial metabolism which is a new area that is now beginning to be explored and targeted for its role in heart disease.



Structure of Guanosine 3', 5' - cyclic monophosphate : Nitric oxide activates soluble guanylyl cyclase (sGC) by binding of NO to heme and non-heme sites of sGC. Two-step activation process of NO binding results in two distinct NO-bound forms of sGC which are characterized by low and high enzymatic activity. Initially NO forms an inactive but NO-responsive six coordinate nitrosyl intermediate by binding to the ferrous, five-coordinate heme moiety of sGC. Further NO binding results in conversion of nitrosyl intermediate sGC species into five-coordinate nitroxyl complex in the presence of magnesium, cGMP and pyrophosphate (11-13). This second NO-binding step accelerates the basal rate of conversion of GTP to cGMP by several hundred folds by breaking the bond between the heme iron and the protein histidine axial ligand results in a conformational change in the catalytic domain of the enzyme (Figure 1). However, in the absence of magnesium, cGMP, pyrophosphate, NO binding to the six-coordinate nitrosyl intermediate sGC species does not activate the enzyme. Thus, at low levels of NO, sGC remains in a low-activity state, whereas at high levels of NO and substrates/products, even the low-activity state sGC can be converted to the highly active state.

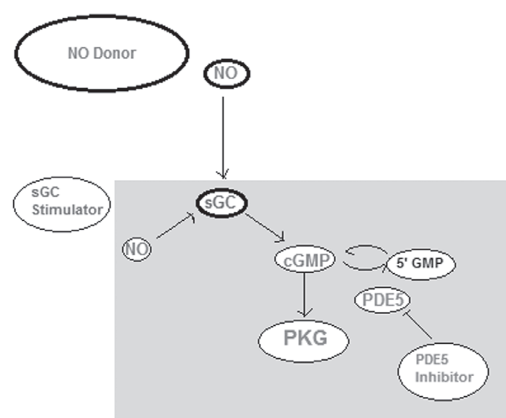


Fig. 1: cGMP-PKG activation by Nitric Oxide (NO).

As initially suggested by the study that the rate of NO dissociation from sGC is much slower than the rate of sGC deactivation so NO can also activate sGC by binding to a non-heme site (14). Again the initial step of sGC activation involves NO binding to the ferrous heme moiety of sGC. However, the second NO binding episode involves binding of NO at a non-heme site, which ruptures the histidine-iron bond and completely activates sGC. At low NO levels, NO dissociates from the non-heme site to give a low-activity state of sGC. The above mechanism of non-heme NO-binding is responsible for cascade of activity by which rapid rise in cGMP production though acute increase in NO. At continual low levels of NO, sGC produces cGMP at long-lasting, low levels. So in this regard the compounds having ability

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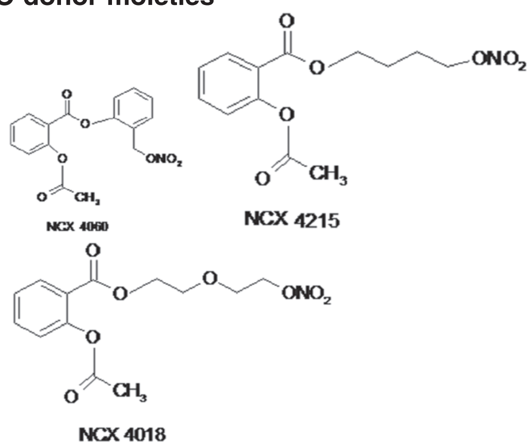
to release NO, which will bind to the heme or nonheme part of the enzyme is very important therapeutic application to cure vascular muscle related disorders. It was observed that cGMP-Dependent Protein Kinase I may be involved for smooth muscle relaxation (15). The compounds such as organic nitrates, nitrites and COX-inhibiniting nitric oxide donators (CINODs) are in clinical trial (16). Following are some examples of compounds, releases NO and causes vasorelaxation.

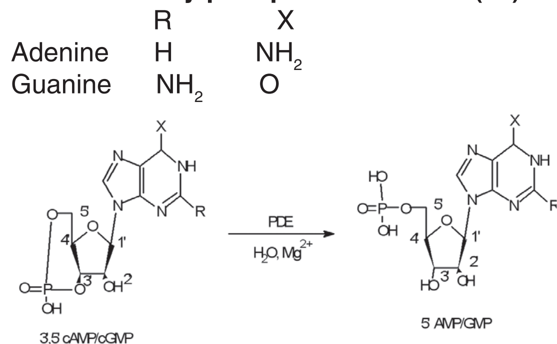
NO donor moieties



Phosphodiesterases: Vascular smooth muscle responses to cGMP-dependent vasodilatory stimuli are regulated by the activity of vascular smooth muscle phosphodiesterase, which catalyzes hydrolyzation of cGMP to inactive products.

Hydrolysis of cyclic nucleotides to nucleotides by phosphodiesterases (17).

	R	X
Adenine	H	NH_2
Guanine	NH_2	O



There are 12 isozymes of phosphodiesterases have been identified in mammalian tissues. Out of these type 5 phosphodiesterase (PDE5) is the predominant isozyme that contributes to regulation of cGMP content in vascular smooth muscle (18, 19). Sildenafil is a specific inhibitor of PDE5 that has been approved for the management of erectile dysfunction in humans (20–22). There is less information about the effect of PDE5 inhibition with sildenafil on endothelium-dependent vasodilation in patients with heart failure.

Nitric oxide (NO) is constitutively produced in the lung by NO synthases from vascular endothelium and the airway epithelia (23 - 24). Depending on alveolar ventilation local NO production regulates pulmonary perfusion to assure optimized ventilation distribution (25 - 26). Nitric oxide synthase activity is regulated on transcriptional and posttranslational redox-based modulation level (27). NO, prostaglandins and natriuretic peptides activates common signalling pathway of endogenous vasodilators such as cyclic adenylate monophosphate [cAMP] and cyclic guanylate monophosphate [cGMP]). Phosphodiesterases (PDEs) represent a superfamily of enzymes, with PDE-1 through PDE-12 being currently known, that inactivate cAMP and cGMP, with different tissue distribution and substrate specificities (28-29). Due to the stabilization of these second messengers, PDE inhibitors differentially regulate levels of cAMP and/or cGMP, depending on their selectivity profile. Therefore, they might offer as therapeutic tools to boost and prolong prostanoid- and NO-related vascular effects. The efficacy of this approach has been proven in several experimental studies (30, 31). Interestingly, the major cGMP-degrading PDE and PDE5 are abundantly present in lung tissue (29). PDE5A was the first cGMP-selective PDE to be discovered and is also activated by cGMP, which binds to its GAF regulatory domain. It is expressed in vascular smooth muscle, endothelium, and fibroblasts.

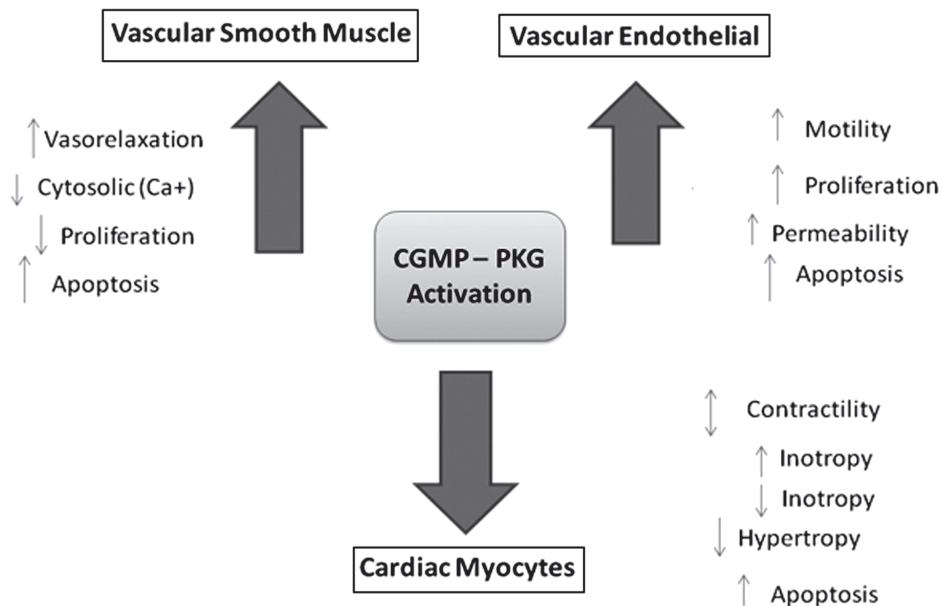


Fig. 2. Physiological effects of cGMP-PKG activation in various cell types of the cardiovascular system.

In this regards, there is a need to investigate more molecules specifically targeting PDE/PDE5/PDE5A for its inhibitory potential to obtain a precise structure and phenomenon for vasorelaxation of smooth muscle. Inhibition of PDE5A is the main target for binding of drug molecule to produce smooth muscle relaxation potency.

Calcium Channel: Calcium channel blockers are widely prescribed drugs for the management of cardiovascular disease and have been approved by regulatory authorities for the treatment of hypertension and symptomatic relief of angina pectoris. Congestive heart failure is an increasingly common syndrome that continues to be a major cause of morbidity and mortality despite current therapy. Many patients with heart failure are treated with calcium blockers as hypertension and coronary disease are the two most common causes of heart failure.

Calcium channels can be divided into two principal subtypes: voltage-activated channels of the sarcolemma and calcium release channels of the sarcoplasmic reticulum. The voltage-activated channels comprise L, T, N, P, Q and R subtypes. The N, P, Q and R type channels are largely found in the nervous system. Voltage-activated L-type calcium channels are present in abundance in myocardial cells and vascular smooth muscle. Muscle contraction happens by entry of calcium through the L-channel. This channel is made up of five subunits termed $\alpha1$, $\alpha2$, β, γ and δ. $\alpha1$ subunit contains the calcium channel conducting pore and binding site for calcium channel blockers. It consists of four repeating hydrophobic motifs (I-IV), each consisting of six segments (S1-S6) that are very similar and span the membrane. Each of the four motifs, which are folded in on them contributes to the calcium channel (32 - 33). Beta-adrenergic stimulation promotes phosphorylation of the $\alpha1$ subunit intracellularly, increasing the probability of channel opening (34).

Binding Sites for Calcium Antagonists: Each of the three classes of calcium antagonist has a

different binding site, located on the alpha subunit. For dihydropyridines, it is on the extracellular loop of the S6 segments (35). Diltiazem also binds on the S6 segments, but not in the extracellular loop (36) and verapamil blocks the calcium channel from the intracellular side (37). The dihydropyridine receptor is the best defined, and numerous dihydropyridine analogues have been synthesized such as nitrendipine or nicardipine. These drugs can activate the receptor and have positive inotropism at low doses, but negative inotropism at high doses (38). Analogues of verapamil, such as anipamil and tiapamil, do not have the same intrinsic mechanisms of action as verapamil (39 - 40). The different binding sites of the three classes help explain their differing tissue selectivities. Because the binding sites of verapamil and diltiazem are intracellular, they only gain access when the channel is open (41).

Immunocytochemical and in situ hybridization studies have made known the presence of the Ca2:calmodulin dependent NO synthase (NOS) in gonadotrophs and folliculostellate cells of the rat anterior pituitary gland. This fact is also similar in normal and neoplastic human pituitaries (42 - 44). A local regulatory function of NO has been recommended, as it can inhibit in vitro prolactin release and moderately mediate the inhibitory effect of dopamine and GABA on prolactin secretion (45). Furthermore, NO also modulates LH, growth hormone and ACTH release (46). NO has also been shown to induce intracellular calcium changes in different cells such as endothelial cells, intestinal epithelial cells, pulmonary artery and smooth muscle cells.

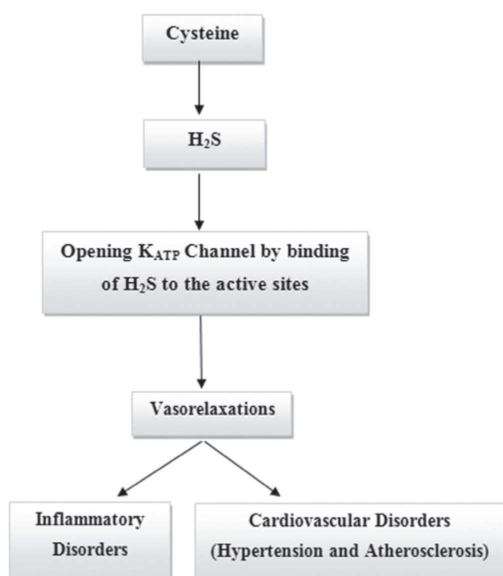


Fig. 3. Vasodilatory effect of H_2S in vascular smooth muscle and tissues.

Keeping in view the findings reported above there is a scope to target calcium channel blocking action by nitric oxide donor drug moieties. There is need to develop correlation of pharmacological action between the different subunits of calcium channel and NO for smooth muscle relaxation potency.

Potassium Channel: Potassium channels, which are the main determinants of resting membrane potential (RP), have been emerged as possible mediators of NO-evoked hyperpolarisation and vasodilatation (47 - 48). Vascular smooth muscle cells and endothelial cells have been found to express at least five types of K+ channels depending on voltage dependence, activation and inactivation kinetics, sensitivity to regulating ions, toxins or other chemical factors. They include a voltage-dependent ATP-sensitive channel (KATP), a depolarization-activated channel (KV), a fast inactivating channel both blocked and an inwardly rectifying channel (Ki), a Ca^{2+}-activated channel (KCa) and apamin (49-51).

There is a contradiction in different reports about the type of K+ channels activated by NO is heterogeneous with vascular beds and animal species. For instance, NO activates K_{Ca} and relaxes the vascular smooth muscles of rabbit aorta and rat mesenteric arteries (52 - 53). But the K_{Ca} is in fact not involved in vasodilatation responses of the newborn pig pia arterioles to NO donor or hypoxia (54). It is also reported that

NO hyperpolarizes the vascular smooth muscles by opening of K_{ATP} channels in rabbit and rat mesenteric arteries (55). In rat small mesenteric artery, NO activates both K_{Ca} and K_{ATP} (56), in rat aorta, the NO donor failed to activate K_{ATP} (57).

H_2S relaxes vascular smooth muscle both in vitro and in vivo probably by opening vascular smooth muscle K_{+ATP} channels through Kv1 (58 - 59). The figure 3 explains the mechanism of H_2S release in the smooth muscle and its therapeutic implications (60). In rats with experimentally induced hypoxic pulmonary hypertension the reduced expression and activity of Cystathionine-g-lyase CSE coupled with a decrease in plasma H_2S concentration was observed in lung tissue recently (61).

Conclusion

The above information and recent studies have clearly shown that the gaseous mediators such as nitric oxide(NO) and hydrogen sulphide (H_2S) are to be the next molecular targets for vascular muscle relaxation action. The potency and role of NO and H_2S as a vasodilator in several clinical conditions makes them an attractive therapeutic target for many conditions. These molecular targets are to be studied and further research is needed to characterize the molecular mechanisms by which NO and H_2S exerts these actions, including their interaction with other molecules in the cell. There is need to investigate H_2S as a vasodilator in humans. The new molecules having a potency to donate NO and H_2S should be synthesized and preclinical and clinical study should be performed. It is hoped that these molecular targets would help to address several unanswered questions in health and diseases.

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NEWS ITEM

Scientific findings

Treatment of Keratitis Eye Infection through biocompatible nanoparticles

For treatment of keratitis, a team of scientists at CCMB has developed a formulation, which is a major fungal eye infection, common among farmers in rural areas. It was a smart polymer-based biocompatible nano particles carrying a formulation that is designed to handle problems caused by fungal infection effectively. Farmers are more likely to contract keratitis, during harvest season. The disease spreads when the farmers come in contact with the leaves of the plants. The fungi reach cornea (outer most part of the eye) and cause inflamation. It then releases enzymes to break down the cornea and use its components as nutrients. The host also responds by its defence mechanism. The infection will eventually lead to cornea damage, which can end up causing scar formation, thus compromising the vision.

New chip-based technology to detect Ebola virus

Researchers have developed a chip-based technology that can be integrated into a portable instrument for use in field situations where rapid, accurate detection of Ebola infections controls and outbreaks. Laboratory tests using preparations of Ebola virus and other hemorrhagic fever viruses showed that the system has the sensitivity and specificity needed to provide a viable clinical assay, researchers said that the current gold standard for Ebola virus detection relies on a method called polymerase chain reaction (PCR), which involves to amplify the virus's genetic material for detection. Compared to this system, PCR detection is more complex and requires a laboratory setting, said Holger Schmidt, University of California, Santa Cruz.. They collaborated with researchers at Brigham Young University and UC Berkeley to develop the system. Virologists at Texas Biomedical Research Institute in San Antonio prepared the viral samples for testing. The study was published in the journal Scientific Reports.

Antibiotics for Bacterial Infection

Scientists at CCMB have identified a novel potential target for the development of new antibiotics to eliminate bacterial infection. According to the official source, the new antibiotics will eliminate bacterial infection by breaking the bonds of the bacterium. Drug-resistance of bacteria has increased despite advances in antibacterial therapy. Meanwhile, the research and development of new antibiotics has declined over the years. A recent study published by our team describes a novel potential drug target for the development of new antibiotics. All biological cells have a membrane that envelops the cell. Bacteria have a rather tough cell wall made up of substances called peptidoglycan. When a bacterium grows and increases in size, the cell wall will also grow. In order to do this it has to break bonds and allow new materials to be bonded. Scientists have identified the role of breaking these bonds in reshaping the cell wall. Blocking these systems could be a new way of eliminating bacterial infections.

Protein system can detect viral infection, kill cancer cells

MIT engineers have developed a modular system of proteins that can detect a particular DNA sequence in a cell and then trigger a desired response, including killing cancer cells or cells infected with a virus. The technology is based on a type of DNA-binding proteins known as zinc fingers. These proteins can be designed to recognize any DNA sequence. The technologies are out there to engineer proteins to bind to virtually any DNA sequence that one want, the paper was published in *Nature Methods.* We felt that there was a lot of potential in harnessing this designable DNA-binding technology for detection. To create the new system, the researchers needed to link zinc fingers, DNA-binding capability with a consequence - either turning on a fluorescent protein to reveal that the target DNA is present or generating another type of action inside the cell.

Killer T-cell therapy shows promise against leukemia

A cancer-killing therapy that engineers a patient's own immune cells to wipe out chronic lymphocytic leukemia (CLL) has shown long-term success in a handful of people. Wednesday. Experts said the approach is on the cutting edge of a growing field known as immunotherapy, which coaxes the body to kill off cancer and may someday revolutionize oncology by ending the use of toxic chemotherapy. The method, known as CTL019, was developed by the University of Pennsylvania's

Abramson Cancer Center and Perelman School of Medicine, which is now reporting the first long-term results on a group of 14 initial patients. Eight of the adults enrolled in the study (57 percent) responded to the treatment, with four going into long-term remission and the other four experiencing a partial response, said the findings in the journal Science Translational Medicine.

SCIENTIFIC NEWS

India to announce climate commitments on Gandhi Jayanti

India announced on October 2, its Intended Nationally Determined Contributions (INDCs) in the lead up to the Paris climate summit to be held in December, the government indicated in U.N. General Assembly that the country's development goals and the U.N. Sustainable Development Goals (UNSDGs) were the same. The INDCs of countries will form the basis for climate negotiations at the Conference of Parties (CoP) 21 under the U.N. Framework Convention on Climate Change in Paris in December. The U.S. administration has said that climate change would be on the agenda of President Barack Obama's meeting with Mr. Modi on Monday. After a two-day tour of the Silicon Valley in the west coast of the U.S., the Prime Minister will travel back to New York for the meeting.

ASTROSAT, India's unique Space Observatory

The launch of Indian Space Research Organisation's (ISRO) ASTROSAT telescope today will be a shot in the arm for astronomers, particularly those in India. This is the first time India is launching a space observatory. But that is not the only reason why the ASTROSAT telescope is so special. Unlike most other telescopes, the five instruments (payloads) of ASTROSAT can observe a wider variety of wavelengths, from visible light to the ultraviolet and X-ray bands. Even in the X-ray band, it can study both low and high energy X-ray regions of the electromagnetic spectrum. Most other satellites are capable of observing only a narrow range of wavelength band.

Success story by diverting water - Diversion-based irrigation system (DBIS)

How diversion-based irrigation system changed the lives of this Odisha village? The residents of this tiny village tucked into the forested hills of the Eastern Ghats range in R. Udayagiri Block of tribal-dominated Gajapati district are a happy lot. Even as the sceptre of drought looms large over several regions of the State, they are able to irrigate their land on the hill slopes by using water from the perennial streams originating from the hill adjacent to their hamlet. For decades together, the 37 tribal families of Sinising used to grow maize and ragi totally dependent upon rainfall. But their lives have changed for the better since water from the perennial hill stream originating in the upper slopes has been brought to their land through the diversion-based irrigation system (DBIS). Seventy-two-year-old Maheswar Pujari of Sinising has not only been able to grow different crops in his fields since the initiative was implemented in their village by voluntary organisation Institute of Social Action and Research Activities (ISARA) three years ago with support from Mennonite Central Committee, a development agency. Pujari has also added more cultivable area by levelling his land that was lying unused on the hill slopes. Apart from maize and ragi, he now grows paddy, groundnut, turmeric, sweet potato, brinjal, beans, cauliflower and many other vegetables.

30 thousands year old frozen virus still infectious

A 30,000-year-old giant virus discovered deep in the Siberian permafrost is still functional and capable of infecting its host, researchers have found. However, the new virus is not a threat to humans; it infected single-celled amoebas during the Upper Paleolithic, or late Stone Age. Dubbed Mollivirus sibericum, the virus was found in a soil sample about 98 feet below the surface and is member of a new viral family, the fourth such family ever found. M sibericum is wider in diameter than the other giant viruses, at 600 nanometres versus 500. It has a genome of 600,000 base pairs which hold the genetic instructions to create 500 proteins.

Nobel Prizes for 2015 in Science

The Nobel Prize in Physics for 2015 has been conferred to Dr.Takaaki Kajita and Dr.Arthur B. McDonald for the discovery of neutrino oscillations, proved that neutrinos have a mass. The Noble prize in Chemistry has been awarded to Dr. Tomas Lindahl, Paul Modrich and Dr.Aziz Sancar for their discovery on mechanistic studies of DNA repair. The Nobel Prize in Physiology and Medicine for 2015 has been awarded to Dr.William C. Campbell and Dr.Satoshi Ômura, Youyou Tu for their discoveries concerning a novel therapy against infections caused by roundworm parasites and to Dr.Youyou Tu for her discoveries concerning a novel therapy against Malaria.

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.