An International Journal

Nature and Science

ISSN 1545-0740

Volume 7 - Number 5 (Cumulated No. 26), July 1, 2009

Marsland Press

P.O. Box 21126, Lansing, Michigan 48909, the United States

525 Rockaway PKWY, #B44, Brooklyn, New York 11212, the United States

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Nature and Science

ISSN: 1545-0740

Volume 7 - Number 5 (Cumulated No. 26), July 1, 2009

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Purification And Characterization Of Two Forms Of Glutathione S-Transferase From *Bimphlaria Alexandrina* Snails

R.R Hamed[1], Kholoud S. Ramadan[2], M .H .Abdel Aziz[1] and T.M.Maharem[2]
[1]Department of Molecular Biology, Genetic Engineering Division, National Research Centre, Cairo, Egypt.
[2]Department of Biochemistry, Faculty of Science, Ain Shams University, Cairo, Egypt
imankam_2@yahoo.com

ABSTRACT: Glutathione S-transferase (GST) have been purified and characterized from the tissue of *B. alexandrina* snails. The purification was carried out by chromatography on DEAE-cellulose. Five GST multiple forms (one unadsorbed and four adsorbed forms) were obtained. The major adsorbed forms GSTP2 and GSTP3 with the highest activity levels were purified to homogeneity by affinity chromatography Sepharose column. The pH optima for the purified GSTP2 and GSTP3 activity with CDNB as co substrate were at pH 6.5. The K_m values for GSH were 0.51 and 0.29 mM for GSTP2 and GSTP3 forms, respectively, while for CDNB were 0.41 mM for GSTP2 and 0.4 mM for GSTP3. The p*Ka* values were 6.0 and 8.0 for GSTP2 and GSTP3, respectively. The bi-substrate kinetics of the purified *B. alexanderina* GSTP2 and GSTP3 with CDNB and GSH as substrates did not obey Michaelis-Menten equation at most of the pHs tested. Hill plots of GSTP2 and GSTP3 for CDNB were non linear at pH 6.5 with values less than 1.0 at low substrate concentrations and higher than 1.0 at high substrate concentrations. Results may indicate that GSTP2 and GSTP3 display a mixture of positive & negative and non cooperativety. [Nature and Science. 2009;7(5):1-10]. (ISSN: 1545-0740).

Keywords: B. alexanderina, Km, GST, affinity chromatography.

INTRODUCTION

Schistosomiasis is one of the major communicable diseases of public health and socio-economic importance in the developing world. The success of these parasites is a result of their ability to switch rapidly between several different environments including snail tissue, fresh water and mammalian blood **(McKerrow & Salter, 2002)**. Snails are important horticultural and agricultural group of pests in that they act as intermediary hosts of animal as well as human parasites. In Egypt, nine species of snail's intermediate host for parasite were discovered **(Abo-Madyan *et al.*, 2005)**. Some snail species that have medical or veterinary importance in Egypt are *Biomphalaria alexanderina (B. alexanderina*), the intermediate host of *S. mansoni***,** *Bulinus truncatus* (Planorbidae, Bulininae), the intermediate host of *S. haematobium, Lymnea trancutula* (Lymnaeidae) the intermediate host of *Fasiola* and *Physa acuta* (Physidae) which is consider as a biological competitor for other snails **(Lardans & Dissous, 1998).**

It has been recognized that the successful control of the disease should include the control of the intermediate host snails. Several control techniques are now available, these include chemical **(Lowe *et al.*, 2005 and Ansaldo *et al.*, 2006)** cultural **(Teo, 2003)**, physical **(Schûder *et al.*, 2003 and Regoli *et al.*, 2005)** and biological control **(Teo, 2006)**.

Living organisms, from the simple bacteria to higher eukaryotes have developed a system for detoxication to protect important macromolecules from damage caused by reactive compounds that may be intracellular; produced during the metabolic processes or extracellular (xenobiotics), which enter into cells through polluted air, water or even food stuff, where they are continuously exposed to non-nutritional foreign chemical species. There are a variety of protection systems in the cell, including enzymes that can catalyze reactions that convert toxic molecules into harmless products. The modified molecules can be excreted from the organism or reused in the cell by some metabolic pathways. The metabolic detoxication of small molecules is carried out by a family of enzymes; specifically designed for that purpose. These enzymes are called detoxication enzymes **(Jakoby & Habig, 1980 and Armstrong, 1997)**.

The enzymatic detoxification of xenobiotics has been classified into three distinct phases which act in a tightly integrated manner. Phases I and II involve the conversion of a lipophilic, non-polar xenobiotics into a more water-soluble and therefore less toxic metabolite, which can then be eliminated more easily

from the cell (phase III) **(Sheehan *et al.*, 2001).** Phase II enzymes catalyze the conjugation of activated xenobiotics to this endogenous water-soluble substrate, such as reduced glutathione (GSH), UDP-glucuronic acid, sulfate, certain amino acids e.g. glycine **(Mannervik *et al.*, 1989 and Meister, 1989).** The conjugation reaction serves the purpose of decreasing the biological activity and to increase the solubility of the original compound **(Falany, 1991)**. The phase II reactions are catalyzed by a number of different enzymes known as transferases. Quantitatively, conjugation to GSH, which is catalyzed by the glutathione transferases (GSTs; also known as glutathione S-transferases), is the major phase II reaction in many species **(Salinas & Wong, 1999).**

Glutathione S–transferases are mainly cytosolic, multifunctional detoxification enzymes, found in most aerobic eukaryotes and prokaryotes exist as dimeric proteins (homodimers or heterodimers). It was proposed that the soluble enzymes have two active sites per dimer each of which functions independently of the other **(Mannervik, 1985b)**.

The aim of the present work was to purify and characterize GST from the tissue of *B. alexandrina* snails and study the mechanisms of enzyme reaction.

MATERIALS AND METHODS

1. Snails

The snails *Biomphalaria alexandrina* used in the present study were maintained in the laboratory under standard conditions of aeration and temperature (25-30°C). They were fed fresh lettuce leaves and placed in dechlorinated water (aerated in a container for several days prior to being used in the experiments).

2. Chemicals

The reduced glutathione (GSH), Epoxy-activated Sepharose 6B, β-mercaptoethanol (β-ME), diethylaminoethyl (DEAE)-cellulose (DE-53) for chromatography and all resins and reagents for electrophoresis were obtained from Sigma Chemical Co. (St Louis, Mo). 1-chloro-2, 4-dinitrobenzene (CDNB), glutathione disulfide (GSSG) and H_2O_2 were purchased from Fluka Company. Other general chemicals were of the highest purity commercially available.

Protein determination

Protein was determined in the eluted fractions by the method of **Bradford (1976)** using bovine serum albumin as a standard.

Assay of GST

Enzyme activity was assayed by monitoring the change in absorbance, due to thioether formation from the substrate 1-chloro-2, 4-dinitrobenzene (CDNB), at 340 nm and 25°C as described by **Ajele & Afolayan (1992).** The assay reaction mixture contained in a total volume of 1.0 ml; 0.1 M potassium phosphate buffer, pH 6.5, 1.0 mM CDNB in ethanol (final concentration of ethanol less than 4 %), 1.0 mM GSH, and the enzyme solution. The extinction coefficient of product was taken to be 9.6 $mM^{-1}cm^{-1}$.

Preparation of Snail Extract

Snails (separated whole animals) were homogenized using Omni mixer in 20 % (w/v) of 0.1 M potassium phosphate buffer, pH 6.5 for the determination of glutathione transferase activity. The homogenate was then centrifuged at 16,000 g for 15 min. The supernatant was filtered through a plug of glass wool to remove floating lipids. The filtrate was designated crude homogenate and saved at -20°C for further analyses.

Preparation of DEAE -Cellulose Column

Diethylaminoethyl cellulose was treated as recommended by Pharmacia information book.

Preparation of GSH - Sepharose Affinity Matrix

Glutathione was coupled to epoxy - activated Sepharose 6B according to **Simons and Vander Jaget, (1977)**.

Purification of GST from *B. alexanderina* Snails

Unless otherwise stated all steps were performed at 4^0C. Crude extract which prepared as mentioned above was applied directly on DEAE-cellulose column previously equilibrated with 25 mM Tris-HCl buffer, pH 8.0. The adsorbed proteins were eluted using stepwise NaCl gradient in 0.02 M Tris-HCl buffer, pH 8.0. All the equilibration and elution buffers contained 2.0 mM mercaptoethanol. Fractions in 5.0 ml volume were collected at a flow rate of 60 ml/h. Fractions containing enzyme activity were pooled (unadsorbed, P1, P2, P3 and P4) according to their elution order. GSTP2 and GSTP3 DEAE-cellulose fractions were applied separately to a GSH-Sepharose column equilibrated with the same equilibration buffer of the DEAE-cellulose column and developed overnight at a flow rate of 10 ml/h. The enzyme was eluted with 50 mM Tris-HCl buffer, pH 9.6 containing 10 mM GSH. Two milliliter fractions were collected.

Characterization of GSTP2 and GSTP3:

The characterization of the purified two GST isoforms with respect to pH optimum and kinetic properties (effect of pH on Km and V_{max}) was thoroughly investigated and shown in result section.

RESULTS

Purification of GST from *B. alexanderina* Snails

The purification of GST is summarized in Table (1). Chromatography on DEAE-cellulose (Fig 1) produced five peaks of GST; named unadsorbed and adsorbed P1, P2, P3 and P4. Their specific activities were ranged from 0.047 to 0.22 units mg^{-1} protein. GSTP2 and GSTP3 were chosen for further purification using GSH-Sepharose affinity chromatography, Fig. (2).

Characterization of the Purified GSTP2 and GSTP3

The effect of pH on *B. alexanderina* GSTP2 and GSTP3 activities were examined between pH 4.5 and 9.0. GSTP2 and GSTP3 exhibited maximum activity at pH 6.5, Fig. (3).

The effect of substrate concentration on the enzyme reaction rates of GSTP2 and GSTP3 were investigated at 25°C and pH values between 5.5 and 9.0. The results were presented in Tables (2-5). The p*Ka* values were in the range from 6.0 -> 9.0 at low and high GSH and CDNB concentration for GSTP2 and GSTP3, (Table 6).

Hill plots were constructed by plotting v/Vmax-v versus [S] (GSH or CDNB concentrations in mM) on log-log scale at pHs ranging from pH 5.5 to pH 9.0 (graphs not shown). Hill coefficients (n; the slope of the plot of v/Vmax-v versus [S]) for GSTP2 and GSTP3 for GSH and CDNB were calculated and the results are summarized in Table (7).

Table (1): Purification scheme of GST from the tissue of *B.alexanderina* snails

Step		Total protein (mg)	Total activity (Units)*	Specific Activity (U/mg Protein)	Fold Purification	Recovery %
Crude Extract:		312.5	43.00	0.138	1.00	100.00
Chromatography on DEAE – cellulose:						
Unadsorbed	**- ve**	31.2	1.48	0.047	0.34	3.44
0.05 M NaCl	**P (1)**	89.8	6.80	0.076	0.55	15.80
0.075 M NaCl	**P (2)**	39.1	4.37	0.112	0.81	10.16
0.125 M NaCl	**P (3)**	48.4	10.63	0.220	1.59	24.73
0.2 M NaCl	**P (4)**	14.8	2.62	0.177	1.28	6.10
Gel filtration on GSH Sepharose:						
GSTP2		0.26	2.65	10.19	91.00	60.73
GSTP3		0.06	5.28	85.16	387.1	54.49

**One unit of glutathione transferase activity was defined as the amount of enzyme that catalyzes the formation of 1.0 µmole of thioether per min under standard assay conditions.*

Table (2): Kinetic parameters of the purified *B. alexanderina* GSTP2 when GSH was the varied substrate at pH 5.5 – 9.0

pH	- 1/Km		Km		1/Vmax		Vmax	
	Low	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**
5.5	2.34	0.3	0.43	3.33	0.28	0.05	3.57	20.0
6.0	1.66	0.43	0.60	2.33	0.18	0.08	5.56	12.5
6.5	1.98		0.51		0.16		6.25	
7.0	0.45		2.22		0.09		11.1	
7.5	3.34		0.30		0.32		3.13	
8.0	2.90		0.34		0.40		2.50	
8.5	8.43	0.56	0.12	1.79	0.68	0.22	1.47	4.55
9.0	4.25		0.24		0.53		1.89	

Table (3): Kinetic parameters of the purified *B. alexanderina* GSTP2 when CDNB was the varied substrate at pH 5.5 – 9.0

pH	- 1/Km		Km		1/Vmax		Vmax	
	Low	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**
5.5	2.52	0.95	0.40	1.05	0.27	0.18	3.70	5.56
6.0	7.6		0.13		0.28		3.57	
6.5	18.3	2.45	0.05	0.41	0.29	0.19	3.45	5.26
7.0	9.35	5.55	0.11	0.18	0.25	0.22	4.00	4.55
7.5	4.35		0.23		0.37		2.70	
8.0	3.06	0.44	0.33	2.27	0.58	0.19	1.72	5.26
8.5	2.78		0.36		0.49		2.04	
9.0	3.1		0.32		0.51		1.96	

Table (4): Kinetic parameters of the purified *B. alexanderina* GSTP3 when GSH was the varied substrate at pH 5.5 – 9.0

pH	- 1/Km		Km		1/Vmax		Vmax	
	Low	High	Low	High	Low	High	Low	High
5.5	2.15	0.60	0.47	1.67	0.33	0.15	3.03	6.67
6.0	1.55		0.65		0.26		3.85	
6.5	3.45		0.29		0.28		3.57	
7.0	1.75		0.57		0.30		3.33	
7.5	4.52		0.22		0.58		1.72	
8.0	3.94		0.25		0.35		2.86	
8.5	8.14	1.30	0.12	0.77	0.56	0.30	1.79	3.33
9.0	7.36	0.66	0.14	1.52	0.53	0.21	1.89	4.76

Table (5): Kinetic parameters of the purified *B. alexanderina* GSTP3 when CDNB was the varied substrate at pH 5.5 – 9.

pH	- 1/Km		Km		1/Vmax		Vmax	
	Low	High	Low	High	Low	High	Low	High
5.5	3.15		0.32		0.24		4.17	
6.0	8.22		0.12		0.23		4.35	
6.5	27.6	2.48	0.04	0.40	0.23	0.15	4.35	6.67
7.0	27.3	4.08	0.04	0.25	0.24	0.16	4.17	6.25
7.5	2.53		0.40		0.24		4.17	
8.0	3.35	0.42	0.30	2.38	0.50	0.15	2.00	6.67
8.5	2.05		0.49		0.35		2.86	
9.0	2.26		0.44		0.39		2.56	

Table (6): Effect of pH on log V_{max}/ K_m of the purified *B. alexanderina* GSTP2 and GSTP3 at different concentrations of GSH and CDNB

p*Ka*		
	Low	**High**
GSH		
GSTP2	**6.5, 7.5, 8.5**	**6.5, 7.5, >9.0**
GSTP3	**6.5, 8.5**	**6.5, 8.0**
CDNB		
GSTP2	**6.5**	**6.0, 7.0, 8.5**
GSTP3	**6.5, 7.0**	**6.0, 7.0, 8.5**

Table (7): Hill coefficients of the purified *B. alexanderina* GSTP2 and GSTP3 for GSH and CDNB at different pHs

pH	GSTP2				GSTP3			
	GSH		CDNB		GSH		CDNB	
	Low	High	Low	High	Low	High	Low	High
5.5	1.07	1.0	0.97	1.03	0.92	1.03	1.05	
6.0	0.91	1.06	1.03		1.01		1.22	
6.5	1.02		1.13	1.01	1.02		0.86	1.24
7.0	1.0		0.92	1.0	1.04		1.01	1.03
7.5	0.82		1.07		0.96		0.99	
8.0	1.0		1.0	1.05	1.01		1.01	1.02
8.5	1.0	0.64	1.06		1.05	0.83	1.05	
9.0	0.97		1.04		0.94	0.67	1.04	

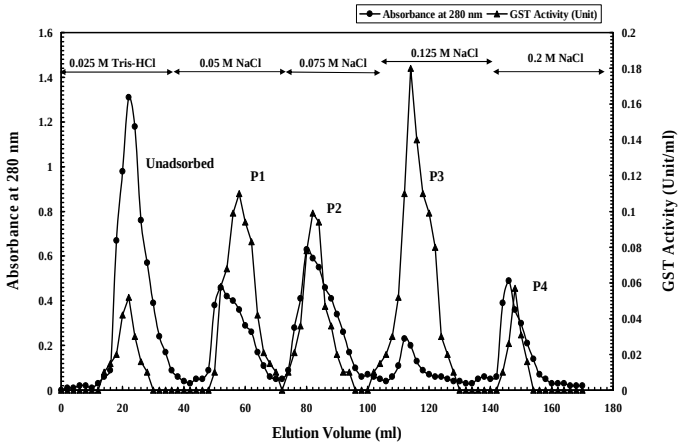

Fig. (1): Typical elution profile of *B. alexanderina* GST chromatography on DEAE-cellulose column (30 x 1.6 cm i.d.) previously equilibrated with 0.02 M Tris-HCl buffer, pH 8.0 containing 2 mM β-mercaptoethanol. Protein was eluted by stepwise NaCl gradient in the equilibration buffer.

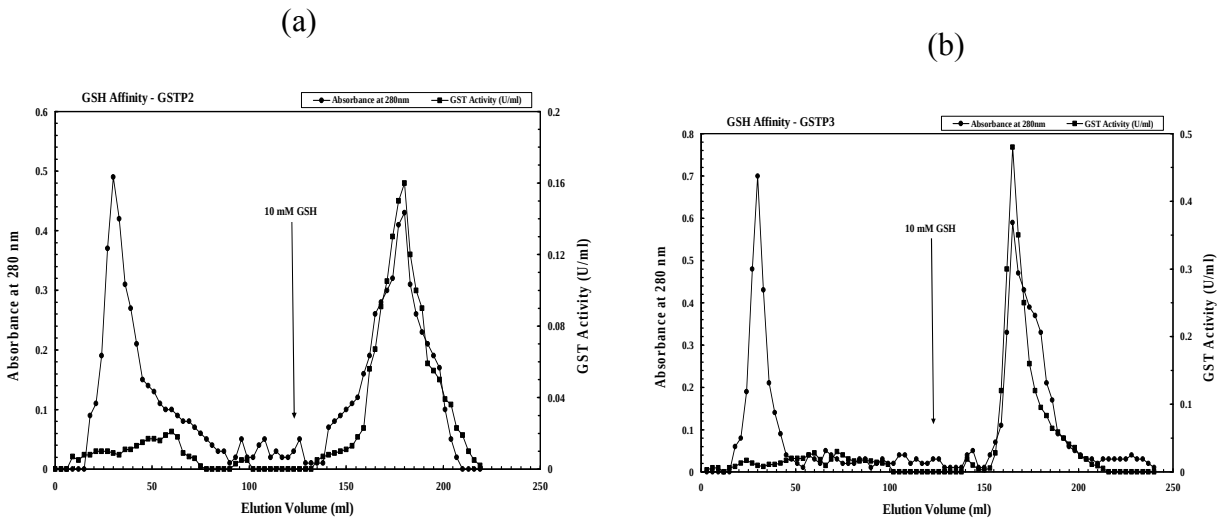

Fig. (2): Typical elution profile for the chromatography of (a) GSTP2 and (b) GST3 on GSH-Sepharose affinity column. The arrow indicates initiation of elution using 10 mM GSH

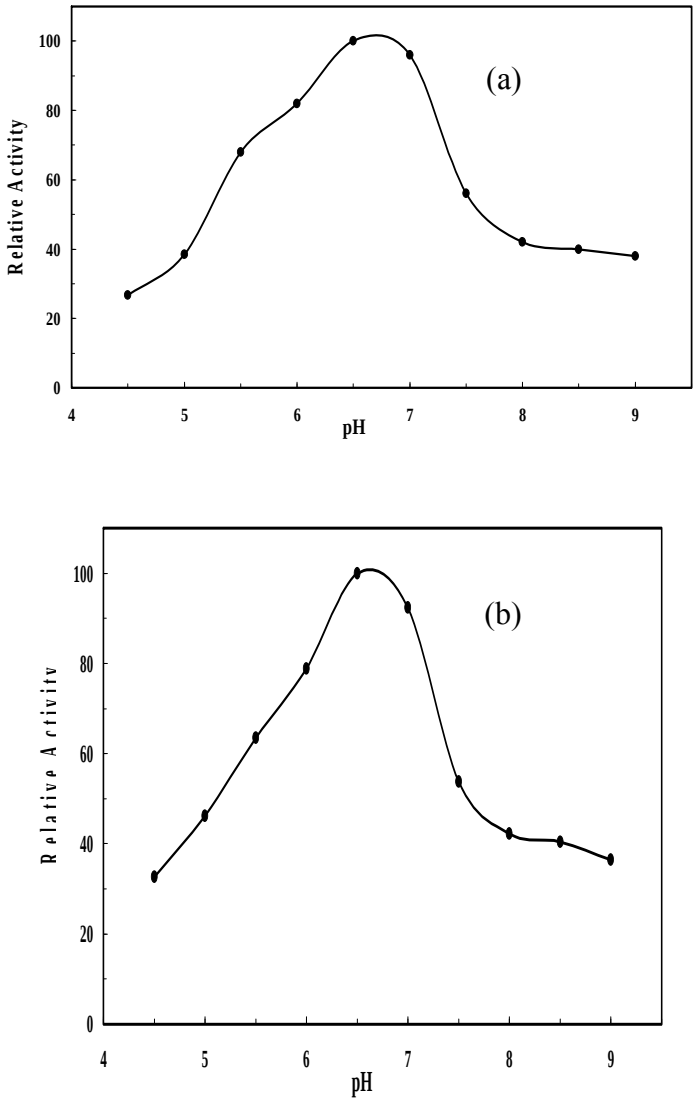

Fig. (3): Effect of pH on the activity of the purified (a) GSTP2 and (b) GSTP3. The buffers used were 0.1 M sodium acetate buffer for pH 4.5 to 5.5, 0.1 M potassium phosphate buffer for pH 6.0 to 8.0 and 0.1 M Tris-HCl buffer for pH 8.5 to 9.0.

DISCUSSION

In the present investigation, chromatographic separation of *B. alexandrina* tissue homogenate on DEAE cellulose indicated the presence of five multiple forms of GST (one unadsorbed and four adsorbed P2, P3, P4 and P5) eluted by NaCl stepwise gradient, **Fig. (1)**. The two major forms, GSTP2 and GSTP3 were further purified on GSH Sepharose according to the method described by **Abdullah (2000)**, The specific activities increased to 10.19 (91-fold purification) and 85.16 unit/ mg protein (387.1-fold purification) with 60.73 and 54.49 % recovery, for GSTP2 and GSTP3 respectively **Fig. (2a &b)**.

Kinetic studies of GSTP2 and GSTP3 of *B. alexanderina* snails, specially the changes in enzyme affinity to its substrates was examined at different pHs. pH optima for GSTP2 and GSTP3 activity with CDNB as co substrate were at pH 6.5, **Fig. (3a &b)**. Similar results were observed for the two GSTs purified from *Bulinus trancatus* **(Abdullah *et al.*, 2004)**.

The bi-substrate kinetics of the purified *B. alexanderina* GSTP2 and GSTP3 with CDNB and GSH as substrates did not obey Michaelis-Menten equation at most of the PHS tested (not shown).

The *Km* values for GSH were 0.51 and 0.29 for GSTP2 and GSTP3 at pH 6.5, respectively, **Table (2, 4)**. These values are comparable to the majority of values reported for invertebrate GSTs. The *Km* values for GSH were 0.23 mM and 0.15 mM for GST2 and GST3 isoenzymes of *B. trancatus*, respectively **(Abdullah *et al.*, 2006)** and 0.19 mM for the mussels *Atactodea striata* **(Yang *et al.*, 2003)**.

The *Km* values for CDNB were calculated with respect to GSTP2 at pH 6.5 to be 0.05 mM at low concentration and 0.41 mM at high concentration, **Table (3)**, while for GSTP3 *Km* values were 0.04 mM and 0.40 mM at low and high CDNB concentration respectively, **Table (5)**.

At the acidic side of the pH, *B. alexanderina* GSTP2 did not obey Michaelis-Menten equation at pH 5.5 and 6.0, for GSH and 5.5 for CDNB. Also GSTP3 exhibited the same behavior at pH 5.5 for GSH **(Fig. not shown)**. At the acid side of pH, one may predict a role for the unfolding of the GST on going to the more acidic side. The presence of the unfolded dimmer, the partially active dimmers may have different *Km* values resulting in the concave behavior for the substrate concentrations.

Results in **Tables (2 – 5)** showed that *Km* values for GSH and CDNB were affected by increasing the pH. The *Km* values for CDNB were decreased from 0.40 mM to 0.05 mM and from 1.05 to 0.41 at low and high CDNB for GSTP2 and from 0.32 mM to 0.04 mM for GSTP3 at low concentration and increased from 0.32 to 0.4 at high concentration by increasing the pH values from 5.5 to 6.5. The same behavior was observed for both GST2 and GST3 of *B. trancatus* isoenzymes, where the *Km* values for CDNB were decreased from 110 μM to 14 μM for GST2 and from 61 μM to 4 μM for GST3 by the increase in the pH from 6.0 to 8.5 **(Abdullah *et al.*, 2006)**. In contrast to the present results **Clark, (1989)**, observed that Michaelis constant for CDNB and DCNB from the insects *Costelytra zealandica* and *Galleria mellonella* were unaffected by increasing the pH from 6.0 to 8.0.

On the other hand, the *Km* values for GSH were increased from 0.43 mM to 0.51 mM at low GSH concentration and decreased from 3.33 mM to 0.51 mM at high GSH concentration, **Table (2)**, with respect to GSTP2. For GSTP3, *Km* values for GSH were decreased from 0.47 and 1.67 at low and high GSH concentration to 0.29 mM, **Table (4)** by increasing pH value from 5.5 to 6.5.

By increasing pH from 6.5 to 9.0, Km for GSH with respect to GSTP2 was decreased from 0.51 to 0.24 at low and high GSH concentrations. With respect to GSTP3 the Km values were decreased from 0.29 mM to 0.14 mM and increased from 0.29 to 1.52 at low and high GSH concentrations. While the *Km* values for CDNB was increased from 0.05 to 0.32 mM and decreased from 0.41 to 0.32 for GSTP2 (Table 3) at low and high CDNB concentrations. With respect to GSTP3, the Km values were increased from 0.04 and 0.4 to 0.44 at low and high CDNB concentrations by changing the pH value from 6.5 to 9.0, (Table 5).

A well documented property of GSTs is the ability to lower the p*Ka* of the sulfhydryl group of the bound GSH. The acidity constant of GSH in the active site ranges between 6.0 – 6.5 for α **(Bjornestedt *et al.*, 1995)**, μ **(Liu *et* al., 1992)** and θ **(Caccuri *et al.*, 1998)** class GSTs.

In the present investigation, the pH dependence of log *Vmax*/*Km*, which reflect the kinetically significant ionizations that occur in the GST-GSH complex suggested apparent p*Ka* values from 6.0 to >9, Table (6). These results suggest that during the catalysis, an amino acid residue with a p*Ka* value of 6.0 must be deprotonated and another residue with a p*Ka* value higher than 9 must be protonated to obtain maximum activity of the enzyme. The p*Ka* value of 6.0 most likely represent that of the thiol group of the bound GSH in the active site of GST. Similar behavior was observed from the kinetic studies of purified *M. mucedo* GST. It indicated that, the fungal GST isoenzymes lowers the p*Ka* of the sulfhydryl group of the GSH as occurs in other GST classes **(Hamed *et al.*, 2005)**.

If the binding of one substrate molecule induces structural or electronic changes that result in altered affinities for vacant sites, the velocity curve will no longer follow Michaelis-Menten kinetics. The phenomenon has been called cooperative binding. Generally, allosteric enzymes yield sigmoidal velocity curves. The binding of one substrate molecule facilitates the binding of the next substrate molecule by increasing the affinities of the vacant binding sites (positive cooperativity)**.** Negative cooperativity occurs when binding of each substrate molecule decreases the intrinsic affinities of the vacant sites. The velocity curve raises rapidly at low substrate concentrations, but then slopes off markedly as the affinities of the

vacant sites decreases. The curve is not sigmoidal. The reciprocal plot is non linear. The linear region at high 1/S values corresponds to the normal hyperbolic saturation of the first (normal affinity) site at low substrate concentrations. As the substrate concentration increases, the second, third and fourth sites fill (with low affinities). The reciprocal plot and scatchard plot are indistinguishes from the plots obtained for a mixture of enzymes with different substrate affinities, or one enzyme with multiple sites of different affinities. The Hill plot has a slope less than 1.0, but approaches a slope of 1.0 at very high and very low substrate concentrations **(Segel, 1993)**.

It was observed that GSTP2 exhibited a non cooperativity at most pHs under investigation at low and high concentrations of GSH and CDNB, except positive cooperativity (n = 1.13), at low CDNB concentration at pH 6.5, and negative cooperativity (n = 0.92) at pH 7.0. Regarding GSH, GSTP2 exhibited negative cooperativity at pH 6.0 at low GSH concentration (n = 0.91) also, at pH 7.5 (n = 0.82) at low and high GSH concentrations as well as at pH 8.5 at high GSH concentration (n = 0.64), **Table (7)**. Nearly the same behaviors were observed for GSTP3, it exhibited positive cooperativity at pH 6.0 (n = 1.22) and at pH 6.5 (n = 1.24) at high CDNB concentration, while at low concentration it exhibited negative cooperativity (n = 0.86). With respect to GSH, GSTP3 showed negative cooperativity (n = 0.92 and 0.94) at pH 5.5 and 9.0 at low GSH concentration and at pH 8.5 (n = 0.83) and pH 9.0 (n = 0.67) and high GSH concentrations, **Table (7)**.

This behavior may suggest that GSTP2 and GSTP3 display a mixture of none, positive and negative cooperativety in the binding of a ligand as though the binding of one substrate molecules induces structural or electronic changes that result in altered affinities for the vacant sites **(Segel, 1993)**. Such behavior has been observed in the sorghum GST B1/B2 **(Gronwald & Plaisance, 1998)**, GST2-2 **(Caccuri *et al.*, 2001)** and the fungus *M. mucedo* GST **(Hamed *et al.*, 2005)**.

REFFERENCES

Abdullah AM (2000): Biochemical studies on glutathione and related enzymes in fresh water snails. Ph.D. thesis, Faculty of Science, Cairo University, Egypt.

Abdullah AM, El-Mogy MA, Farid NM and El-Sharabasy MM (2004): Purification and characterization of glutathione transferases from *Bulinus trancatus*. J. of Genetic Eng. & Biotechnol (NRC). 2: 73 - 87.

Abdullah AM, El-Mogy MA, Farid NM and El-Sharabasy MM (2006): Two glutathione S-transferase isoenzymes purified from *Bulinus truncates* (Gastropoda: Planorbidae). Comp. Biochem. Physiol. 143 (C): 76 - 84.

Abo-Madyan AA, Morsy TA, Motawea SM, El Garhy MF and Massoud AM (2005): Spot light survey on fresh-water snails of medical importance in Al Fayoum Governorate, Egypt. J. Egypt Soc. Parasitol. 35 (1): 49 - 58.

Ajele JO and Afolayan A (1992): Purification and characterization of glutathione transferase from the giant African snail, *Archachatina marginata*. Comp. Biochem. Physiol. 103 (B): 47 - 55.

Ansaldo M, Nahabedian DE, Holmes-Brown E, Agote M Ansary CV, Guerrero NR and Wider EA (2006): Potential use of glycogen level as biomarker of chemical stress in *Biomphalaria glabrata*. Toxicology. 5, 224 (1-2): 119-27.

Armstrong RN (1997): Structure, catalytic mechanism and evolution of the glutathione transferases. Chem. Res. Toxicol.10: 2-18.

Bjornestedt R, Stenberg G, Widersten M, Board PG, Sinning I, Jones TA and Mannervik B (1995): Functional significance of arginine 15 in the active site of human class Alpha glutathione transferase A1-1. J. Mol. Biol. 247: 765 - 773.

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248 - 254.

Caccuri AM, Antonini G, Board PG, Flanagan J, Parker MW, Paolesse R, Turella P, Chelvanayagam G and Ricci G (2001): Human glutathione transferase T2-2 discloses some evolutionary strategies for optimization of the catalytic activity of glutathione transferases. J. Biol. Chem. 276, 5432 - 5437.

Caccuri AM, Lo Bello M, Nuccetelli M, Nicotra M, Rossi P, Antonini G, Federici G and Ricci G (1998): Proton release upon glutathione binding to glutathione transferase P1-1: Kinetic analysis of a multistep glutathione binding process. Biochemistry 37: 3028 - 3034.

Clark AG (1989): The comparative enzymology of the glutathione S-transferases from non-vertebrate organisms. Comp. Biochem. Physiol. 92 (B): 419 - 446.

Falany CN (1991): Molecular enzymology of human liver cytosolic sulfotransferases. Trends in Pharmacology. Sci. 12: 255 - 259.

Gronwald JW and Plaisance KL (1998): Isolation and characterization of glutathione S-transferase isozymes from sorghum. Plant Physiol. 117: 877 - 892.

Hamed RR, Abu-Shady MR, El-Beih FM, Abdullah AM and Afifi OM (2005): Purification and characterization of a glutathione S-transferase from *Mucor mucedo*. Pol J Microbiol. 54(2):153 - 160.

Jakoby WB and Habig WH (1980): In "Enzymatic Basis of Detoxification" Vol. 2, pp: 63 - 94, (Ed. Jakoby, W. B.) Academic Press, New York.

Lardans V and Dissous C (1998): Snail control strategies for reduction of Schistosomiasis transmission. Parasitol Today. 14 (10): 413 - 417.

Lowe D, Xi J, Meng X, Wu Z, Qui D and Spear R (2005): Transport of *Schistosoma japonicum* cercariae and the feasibility of niclosamide for cercariae control. Parasitol. Int. 54 (1): 83 - 89.

Liu S, Zhang P, Ji X, Johnson WW, Gilliland GL and Armstrong RN (1992): Contribution of tyrosine 6 to the catalytic mechanism of isoenzyme 3-3 of glutathione S-transferase. J. Biol. Chem. 267: 4296 - 4299.

Mannervik B (1985b): The isoenzymes of glutathione transferase. Adv. Enzymol. Rel. Areas Mol. Biol. 57: 357 - 417.

Mannervik B, Carlberg I and Larson K (1989): Glutathione; general review of mechanism of action. In "Coenzymes and cofactors" Vol. III A, pp: 475 - 516 (Eds. Dolphin, D. Poulson, R. and Avramovic, O.) John Wiley and Sons, New York.

McKerrow JH and Salter J (2002): Invasion of skin by Schistosoma cercariae. Trends Parasitol. 18 (5): 193 - 195.

Mannervik B, Carlberg I and Larson K (1989): Glutathione; general review of mechanism of action. In "Coenzymes and cofactors" Vol. III A, pp: 475 - 516 (Eds. Dolphin, D. Poulson, R. and Avramovic, O.) John Wiley and Sons, New York.

Regoli F, Gorbi S, Machella N, Tedesco S, Benedetti M, Bocchetti R, Notti A, Fattorini D, Piva F and Principato G (2005): Pro-oxidant effects of extremely low frequency electromagnetic fields in the land snail *Helix aspersa*. Free Radic. Biol. Med. 39 (12): 1620 - 1628.

Salinas AE and Wong MG (1999): Glutathione S-transferases. Curr. Med. Chem. 6: 279 - 309.

Schûder I, Port G and Bennison J (2003): Barriers, repellents and antifeedants for slug and snail control. Crop Protection 22 (8): 1033 - 1038.

Segel IH (1993): Multisite and allosteric enzymes. In "Enzyme kinetics, behavior and analysis of rapid equilibrium and steady-state enzyme systems" pp: 346 - 464 (Eds. John Wiley) Sons Inc. New York.

Sheehan D, Meade G, Foley VM and Dowd CA (2001): Structure, function and evolution of glutathione transferases: Implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem. J. 360: 1 - 16.

Simons PC and Vander Jaget DL (1977): Purification of glutathione S- transferase from human liver by glutathione-affinity chromatography. Anal. Biochem. 82: 334 - 341.

Teo SS (2003): Damage potential of the golden apple snail *Pomacea canaliculata* (Lamarck) in irrigated rice and its control by cultural approaches. Int. J. Pest Manage. 49: 49 - 55.

Teo SS (2006): Evaluation of different species of fish for biological control of golden apple snail *Pomacea canaliculata* (Lamarck) in rice. Crop Protection. 25: 1004 - 1012

Yang Hai-ling, Qing-yin Zeng, Li-jia Nie, Sheng-geng Zhu and Xian-wan Zhoua (2003): Purification and characterization of a novel glutathione S-transferase from *Atactodea striataq*. Biochem. Biophys. Res. Comm. 307: 626 – 631.

3/22/2009

Study of Gastro Esophageal Reflux Disease in COPD Patients

Adel M.Khattab*, Khaled Z. El-Karmouty**,Tamer M. Aly*, Asmaa H. Abdel Azeem*

*Chest Department, Faculty of Medicine, Ain Shams University
**Internal Medicine Department, Faculty of Medicine, Ain Shams University

imankam_2@yahoo.com

Abstract: This study included 30 COPD inpatients in chest and internal medicine departments in Ain Shams University Hospital , 15 of them with moderate COPD and 15 of them with severe COPD (by Gold 2007)[1], 25 were males and 5 were females (active or passive smokers). The aim of the work was to study the prevalence of GERD in COPD patients and its effect on the number of exacerbations of COPD. Both groups were subjected to history taking, full clinical examination, full laboratory investigations, radiography, spirometry, arterial blood gases and upper GIT endoscopy and biopsy. Results revealed that the prevalence of GERD in COPD patients was 53.3% in the moderate group,73.3 in the severe group(total= 63.3 %) by endoscopy & was 66.6% in the moderate group,93.3 % in the severe group ((total= 80 %) by biopsy being more prevalent in the severe group of COPD. GERD severity increases as the degree of COPD increases (there were more patients with advanced grades among severe COPD than the moderate group). GERD increases with increase in the smoking (pack/year) both in moderate & in the severe groups. Moreover, there were increase in the frequency of exacerbations of COPD in GERD patients both in moderate & in the severe groups. From this study we conclude that GERD is common in COPD patients being more among severe COPD. Also GERD increases the number of exacerbations of COPD. [Nature and Science. 2009;7(5):11-18]. (ISSN: 1545-0740).

Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of chronic morbidity and mortality throughout the world; it is the fourth leading cause of death in the world[2]. COPD is a preventable and treatable disease with some significant extra pulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles and gases[1].Gastro esophageal reflux disease (GERD) is the collective term used to describe abnormal reflux of gastric content into the esophagus as well as the symptoms and mucosal disease associated with it. Clinical manifestations of GERD include heart burn, regurgitation, dysphagia, chest pain, cough and other esophageal symptoms. GERD is known to cause errosive esophagitis and Barrette esophagus .Currently upper GIT endoscopy is the main clinical tool for visualizing esophageal lesions[3].

Micro aspiration of gastric contents and/or vagal nerve induced bronchospasm from gastric acid irritation of the esophagus may contribute to the observed association between GERD and pulmonary disease or symptoms[4].

Gastroesophageal reflux disease (GERD) may cause, trigger, or exacerbate many pulmonary diseases. The physiologic link between GERD and pulmonary diseases has been extensively studied in asthma; however, in other pulmonary diseases, including interstitial pulmonary fibrosis (IPF), cystic fibrosis and COPD, the link has been less well studied[5].

The prevalence of reflux symptoms is related to the degree of obstruction of airflow in patients with COPD[6].

Subjects and Methods:

This work was conducted in chest and internal medicine units of Ain- shams university Hospital.

This study included 30 subjects with established diagnosis of COPD based on the criteria stated by Gold 2007 for diagnosis of COPD, 25of them were males and 5 were females (active or passive smokers) . There exclusion criteria were:

1) Respiratory disorders other than COPD(such as asthma or IPF)
2) Known esophageal disease such as cancer, achalasia, stricture or active peptic ulcer.

All were subjected to the following:

1) Full history taking (questionaire is included in the sheet) and clinical examination.

2) Routine laboratory investigations:

1 Random blood sugar (RBS).
2 Liver function tests (AST, ALT, total and direct bilirubin, total proteins and albumin.).
3 Kidney function tests (BUN, creatinine).
4 Complete blood count (CBC).
5 Prothrombin time and partial thromboplastin time (PT and PTT).

3) Chest X ray (postero- anterior view)

4) Arterial blood gases analysis (ABG) with particular attention to PH, PaCO2 & PaO2 and O2 saturation.

5) Pulmonary function tests: in the form of spirometry including forced vital capacity (FVC) expressed as percent predicted, forced expiratory volume in 1st second (FEV1) expressed as percent predicted, ratio of FEV1 to FVC expressed as a percentage and average flow rate between 25% and 75% of the FVC (FEF 25 – 75).

6) Upper GIT endoscopy and biopsy showed mucosal changes at the lower end of the esophagus, described by:

Los Angels Criteria for classification of GERD[(6)]:

Grade	Description
A	one (or more) mucosal break not longer than 5mm that does not extend between the tops of two mucosal folds
B	one (or more) mucosal break more than 5mm long that does not extend between the tops of two mucosal folds
C	one (or more) mucosal break that is continuous between the tops of two or more mucosal folds, but which involves less than 75% of the circumference
D	one (or more) mucosal break which involves at least 76 % of the esophageal circumference

Analysis of data was done by IBM computer using SPSS (statistical program for social science version 12).

Results

This study included 30 subjects divided into 2 groups:

Group 1: 15 COPD patients with moderate chronic obstructive pulmonary disease.

Table (1) Description of different variables among moderate group

Variables	Mean ±SD	Range
Age	62.6±12	46-84
Gender Male Female	 12 3	 80% 20%
Smoking (pack/year)	25±11	10-50
FVC%	87±15	64-116
FEV1%	62±7	51-79
FEV1/FVC	60.7±5.8	51-70
FEF25-75%	36.2±13.6	15-56
Duration of COPD in years	5.6±3	2-12

Group 2: 15 COPD patients with severe chronic obstructive pulmonary disease**.**

Table (2) Description of different variables among severe group

Variables	Mean ±SD	Range
Age	55.6±8	40-68
Gender Male Female	 13 2	 86.7% 13.3%
Smoking (pack/year)	37±16	10-60
FVC%	65.2±14	45-92
FEV1%	36±8	22-48
FEV1/FVC	47.6±8	32-64
FEF25-75%	21±10	6-40
Duration of COPD in years	7±5	3-17

Table (3) Comparison between both groups as regard prevalence of GERD

GERD	**Total N=30**	**Moderate N=15**	**Severe** N=15	**P**
No	**11(36.7%)**	7(46.7%)	4(26.7%)	
Yes	**19(63.3%)**	8(53.3%)	11(73.3%)	>0.05 NS

This table shows no statistically significant difference could be detected between both groups as regard GERD by using Fisher exact test. .however ,the number of GERD patients among severe COPD is more than that in the moderate group & the overall prevalence of GERD in COPD patients (by endoscopy) in the taken sample was 63.3% .

Table (4) Comparison between both groups as regard GERD grades

GERD Grades	**Moderate** N=15	**Severe** N=15	X^2	**P**
No	7(46.7%)	4(26.7%)		
Grade A	2(13.3%)	3(20%)	1.8	>0.05 NS
Grade B	4(26.7%)	4(26.7%)		
Grade C	1(6.7%)	2(13.3%)		
Grade D	1(6.7%)	2(13.3%)		

This table shows no statistically significant difference could be detected between both groups as regard GERD grades by using chi-square test.

Table (5) Comparison between GERD and non GERD cases as regard different variables among moderate group

Variables	**No GERD** N=7	**GERD** N=8	**t**	**P**
Age	60±9	64±14	0.7	>0.05

BMI	25.8±4	30±5	1.8	>0.05
FVC	85.6±7	88.7±20	0.4	>0.05
FEV1	64.4±3.9	60±9	1	>0.05
FEV1/FVC	60±5	60.6±6	0.03	>0.05

BMI: body mass index

This table shows no statistically significant difference could be detected between both groups (non GERD & GERD)among moderate COPD patients as regard different variables by using unpaired t-test.

Table (6) Comparison between GERD and non GERD cases as regard different variables among severe group

Variables	**No GERD** N=4	**GERD** N=11	**t**	**P**
Age	57.8±5.6	54.8±9	0.6	>0.05
BMI	25±5	28.7±6	1.02	>0.05
FVC	69±14	52.5±8	**2.3**	**<0.05 S**
FEV1	38±6	32±11	1.3	>0.05
FEV1/FVC	49±10.5	47±7.5	0.6	>0.05

This table shows that FVC was lower among GERD cases with statistically significant difference in between both groups (non GERD & GERD) among severe COPD patients by using unpaired t-test. on the other hand there is no significant difference as regard other variables.

Table (7) Comparison between GERD and non GERD cases as regard smoking (pack / year) among moderate group

Smoking (pack /year)	**No GERD** N=7	**GERD** N=8	**t**	**P**
Mean ±SD	16.7±4	32.1±4	**3.2**	**<0.01 HS**

This table shows that smoking (pack/year) was higher among GERD cases with statistically highly significant difference in between both groups by using unpaired t-test.

Table (8) Comparison between GERD and non GERD cases as regard smoking (pack / year) among severe group

Smoking (pack /year)	**No GERD** N=4	**GERD** N=11	**t**	**P**
Mean ±SD	15±7	42±14	**2.5**	**<0.01 HS**

This table shows that smoking (pack/year) was higher among GERD cases with statistically highly significant difference in between both groups by using unpaired t-test.

Table (9) Comparison between GERD and non GERD cases as regard frequency of exacerbations among moderate group

Frequency of exacerbations	**No GERD** N=7	**GERD** N=8	**t**	**P**
Mean ±SD	**3±0.8**	**5+0.9**	**3**	**<0.01 HS**

This table shows that frequency of exacerbations was higher among GERD cases with statistically highly significant difference in between both groups by using unpaired t-test.

Table (10) Comparison between GERD and non GERD cases as regard frequency of exacerbations among severe group

Frequency of exacerbations	**No GERD** N=4	**GERD** N=11	**t**	**P**
Mean ±SD	3±0.9	4+1.6	**2.1**	**<0.05 S**

This table shows that frequency of exacerbations was higher among GERD cases with statistically significant difference in between both groups by using unpaired t-test.

Table (11) Correlation between GERD grade versus other variables among moderate group

Variables	**GERD grade** r	P	Significance
Age	**0.02**	**>0.05**	**NS**
BMI	**0.44-**	**<0.05**	**S**
Pack/year	**0.87**	**<0.01**	**HS**
FEV1	**0.34**	**>0.05**	**NS**
Number of GERD symptoms /wk	**0.86**	**<0.01**	**HS**
Duration of COPD in years	**0.03**	**>0.05**	**NS**
Frequency of exc. In last year	**0.88**	**<0.01**	**HS**

This table shows highly significant positive correlation between GERD grade versus smoking (pack/year), symptoms/week and frequency of exacerbations of COPD in last year and significant correlation versus BMI by using Spearman correlation test. On the other hand no significant correlation versus other variables.

Table (12) Correlation between GERD grade versus other variables among severe group

Variables	**GERD grade** r	P	Significance
Age	**0.06**	**>0.05**	**NS**
BMI	**0.45-**	**<0.05**	**S**
Pack/year	**0.77**	**<0.01**	**HS**
FEV1	**0.30**	**>0.05**	**NS**
Number of GERD symptoms /wk	**0.80**	**<0.01**	**HS**
Duration of COPD in years	**0.05**	**>0.05**	**NS**
Frequency of exc. In last year	**0.54**	**<0.05**	**S**

This table shows highly significant positive correlation between GERD grade versus smoking (pack/year), symptoms/week and significant correlation versus BMI and frequency of exacerbations by using Spearman correlation test. On the other hand no significant correlation as regard other variables.

Table (13) Comparison between both groups (moderate & severe COPD) as regard different GERD symptoms

Variables	Total	**Moderate** N=15	**Severe** N=15	X^{X2}	*P*
Heart burn				**0.0**	**>0.05 NS**
No	2(6.7%)	1(6.7%)	1(6.7%)		
Yes	28(93.3%)	14(93.3%)	14(93.3%)		
Regurgitation					
No	8(26.7%)	2(13.3%)	6(40%)	2.7	>0.05
Yes	22(73.3%)	13(86.7%)	9(60%)		NS
Vomiting					
No	18(60%)	8(53.3%)	10(66.6%)	3.2	>0.05
Yes	12(40%)	7(46.7%)	5(33.3%)		NS

This table shows no significant difference between both groups as regard different symptoms by using chi-square test. In general ,there was 93% of COPD patients were complaining of heart burn ,73% of regurgitation &40% of vomiting ,other symptoms like haematemesis,melena, and dysphagia were 10%

Table (14) Distribution of both groups as regard biopsy results

Biopsy	**Moderate** N=15	**Severe** N=15	**Total** N=30
Negative	5(33.3%)	1(6.7%)	6(20%)
Positive	10(66.6%)	14(93.3%)	24(80%)

This table shows the prevalence of positivity in both groups (the overall prevalence of GERD by biopsy=80%)

Discussion

Many studies have discussed the impact of gastroesophageal reflux disease on asthma, (especially in children). But few have done regarding COPD. ***Lopes et al (2002)***[8] has documented that only tiny amount of acids is necessary to trigger pronounced symptoms of cough, wheezes and airway obstruction evidenced by physiologic measurements such as spirometry; which may support the explanation of the possible association between GERD and COPD.

The aim of this work is to study the prevalence of GERD in COPD patients and its effect on the

number of exacerbations of COPD that may show a possible modifiable risk factor that by control may improve heath status and decrease the cost of health care and hospitalizations.

In this study 30 patients with known diagnosis of COPD by ***GOLD criteria; (2007)***[1] were chosen from inpatient chest and internal medicine departments of Ain Shams University hospital. Patients were excluded if they have respiratory disorders other than COPD (such as asthma or IPF), or known esophageal disease (such as cancer, achalasia, stricture or active peptic ulcer); they were divided into 2 groups:

Group 1: with moderate COPD (15 patients).

Group 2: with severe COPD (15 patients).

Graded by spirometry. Both were asked to complete questionnaire then both underwent upper GIT endoscopy for assessment of the GERD state.

In this study there was no significant difference between moderate and severe COPD patients and between GERD and non GERD subjects regarding the age and there was no correlation between age and GERD grade either in moderate and in severe patients.

In the present study, there was no significant difference between moderate and severe COPD patients. However it is to some extent lower in severe group which is in agreement with a study done by ***Raafat (2006)***[9] denoting that BMI is lower among COPD patients being more severely lowered in more advanced cases; that might be caused either by chronicity or by steroid induced myopathy which may affect diaphragmatic muscle mass and depresses diaphragmatic contractility . Also there was no significant difference between GERD & non GERD patients. In this study, (in either moderate or severe COPD) there was inverse relation between BMI & GERD grade(i.e. GERD increases while BMI decreases); which is against the fact that obesity is a risk factor for GERD denoted by ***Hampel*** **et al., 2005**[10]. This means that in these COPD patients, the advanced GERD grades may be more related to the severity of COPD rather than obesity.

Regarding smoking (pack/ year), there was significant difference between moderate and severe COPD patients being higher in severe group.Also there was highly significant difference between GERD and non GERD subjects being higher among GERD patients in either the moderate or the severe groups and it was found that in GERD subjects, there was positive highly significant correlation between smoking (pack/ year) and GERD grade. i.e. increase in the smoking (pack/ year), increases the GERD grade; that correlates with the fact that smoking causes marked reduction in LESP by its content of nicotine which may block the cholinergic control mechanism and delay gastric emptying which predispose to gastroesophagel reflux[11].

Considering the frequency of exacerbations of COPD, there was highly significant difference between COPD patients with GERD and COPD patients without GERD being higher among GERD patients in either moderate or severe COPD. Within GERD patients, there was positive highly significant correlation between GERD & frequency of exacerbations of COPD in the moderate group & significant in the severe group; that correlates well with a study done by ***Ivan et al (2006)***[12] based on questionnaire Cross sectional survey on large number (91) outpatient clinics' patients with established diagnosis of COPD that noted that the rate of exacerbations of COPD was twice as high in patients with GERD symptoms compared to those without GERD symptoms. It has the limitations of any cross sectional study; one of them was the recall bias, when patients responded to questions that required the use of long term memory, which is partially overcomed in our study by a confirmatory investigation (upper GIT endoscopy).

Studying the symptomatology of GERD there was no significant difference in GERD symptoms in moderate and severe COPD patients, and the most common complaint was heartburn followed by regurgitation. It was obvious also that not all complaining patients showing evidence of GERD endoscopically. We noticed that 93.3% were complaining of heart burn, for example, while 63.3 % were having GERD endoscopically among COPD patients. This may be explained by the possibility of the presence of non erosive GERD that can't be identified by endoscopy and appears by biopsy which revealed 80 % prevalence.

There was no significant difference as regarding GERD grades in between moderate and severe COPD. However, there were more number of patients with more severe GERD grades in severe than in moderate COPD which means that GERD grade increases with increase severity of COPD.

Few other studies tried to find an association between COPD and GERD; a study done by ***Robert et al (2007***[13] on 41 COPD outpatients with a mean FEV1 OF 24 % (advanced COPD)on their baseline medical regimen at the time of the study, using dual probe 24 h esophageal PH monitoring and manometry revealing that the prevalence of GERD was 57 % and only one third of the patients reported symptoms (heart burn and/ or regurgitation) and concluded that GERD is common in advanced COPD patients who were often asymptomatic and have a relatively high prevalence of isolated abnormal proximal reflux ;but it has the

limitation of that all the patients were advanced COPD patients and the findings might not be applicable to individuals with milder disease. The present study involved moderate & severe cases, the prevalence with endoscopy was ;in the moderate group 53.3 % & in the severe group 73.3 % (total =63.3%) & 93.3 % .60 % were complaining of GERD symptoms (heart burn and regurgitation ,respectively).

A study done by ***Mokhlessi et al (2001)***[14] using GERD questionnaire given to 140 patients and observed a high prevalence of GERD symptoms in patients with COPD with a trend to higher prevalence in severe COPD and increased use of acid suppressive medications among patients with COPD than the control; but this study had a limitation of not having objective measurements of acid reflux.

Upper GIT endoscopy identified only the erosive GERD therefore there may be still a portion of GERD patients who can't be identified by the upper GIT endoscopy(16.7%) and can be identified by biopsy.

Finally GERD is common in COPD patients, proved by upper GIT endoscopy, also GERD grade increases as the degree of obstruction of COPD increases and once diagnosed should be treated and followed for early detection of complications.

Also patients who have COPD and GERD are more likely to have an increased number of COPD exacerbations, when compared to non GERD group.

References:

1- GOLD. Global initiative for chronic Obstructive Lung Disease; Global strategy for diagnosis, management and prevention of chronic obstructive lung disease. Gold Website, WWW.gold COPD.org. 2007;p2,3,33,35,36,37&69.
2- Lopez AD, Shibuya K and Held LS. Chronic obstructive pulmonary disease: current burden and future projections. Eur Respir J 2006; 27(2):397-412.
3- DeVault KR, and Castell DO. Updated guidelines for the diagnosis and treatment of gastroesophageal reflux disease. Am. J Gastrointerol(2005)100:190-200.
4- Weaver EM: Association between gastro esophageal reflux and sinusitis, otitis media, and laryngeal malignancy: a systematic review of the evidence. Am J med 2003;115(suppl3A):81S-89S
5- Susan M and Harding MD. Pulmonary manifestations of GERD: pathophysiology and management, @www. google.com.2004.
6- Andereu M, Delgado S and Seoane A. Gastroesophageal reflux (GER) symptoms in patients with chronic obstructive pulmonary disease(COPD).Gut 2003;52 (suppl.6): A124.
7- Lundell LR, Dent J and Bennett JR. Endoscopic assessment of esophagitis: clinical and functional correlates and further validation of the Los Angels Classification. Gut 1999; 45:172-180.
8- Lopes FD, Alvarenga GS and Quiles R. Pulmonary responses to tracheal or esophageal acidification in guinea pigs with airway inflammation. J Appl Physiol 2002; 93 (3): 842-7.
9- Raafat RH: assessment of nutritional status of COPD and Bronchial asthma patients in relation to severity and duration of the disease.MSC thesis, chest department ,Ain Shams University 2006.
10- Hampel H, Abraham NS and El-Serage HB. Meta-analysis: and the risk for gastro esophageal reflux disease and its complications. Ann. Int. Med. 2005; 143:199-211.
11- Richter JE and Castell DO. Drugs, foods and other substances in the cause and treatment of reflux esophagitis Med. Clin N Am 1981; 65(6):1223-1234.
12- Ivan ER, Mark DO and Peter W. Role of gastroesophageal reflux symptoms in exacerbation of COPD, chest 2006,130:1096-1101.
13- Robert RK, Kay S and Joanne LB. High prevalence of proximal and distal gastroesophageal reflux disease in advanced COPD Chest J 2007; 06-2246 V1.
14- Mokhlesi B, Morris AL and Huang CH. Increased prevalence of gastroesophageal reflux symptoms in patients with COPD. Chest 2001;119:1043-1048.

3/22/2009

Biliary Ki-ras gene Mutation Analysis In Diagnosing Malignant Biliary Stricture

**Khaled Z. Elkarmouty,*Nanees A. Adel, , *Marcel W. Keddes, *Hisham H. Radwan, and ***Mohamed Kamel AboGolayel.

* lecturer of internal medicine, Ain Shams university.

** Assisst Prof of internal medicine Ain Shams university.

*** PHD in medical applications of laser, Medical research center, Ain Shams University

imankam_2@yahoo.com

Abstract

Background: Identifying the nature of biliary stricture whether benign or malignant is a prerequisite for subsequent management. The diagnosis of malignancy is difficult to be achieved with standard imaging or histopathological techniques in many situations. **Aim of the work**: is to evaluate the diagnostic value of detecting the Ki-ras gene mutation compared to bile fluid cytology and brush cytology to differentiate between benign and malignant strictures in a group of Egyptian patients. **Methods**: Ki-ras codon 12 point mutation was identified by PCR and RFLP in bile aspirate obtained during ERCP for 40 patients with biliary stricture (finally diagnosed as benign stricture in 15 patients and as malignant stricture in 25 patients) . Standard cytological examination was also performed on bile bile fluid and on brush cytology samples of the stricture. **Results**: Overall 13 patients (52%) in malignant group had mutant Ki-ras gene and 2 patients (13.3%) in benign group were positive for Ki-ras mutation. For diagnosing malignancy Ki ras gene mutation analysis had 52%sensitivity, 86.6% specificity while brush cytology had 8% sensitivity and 100% specificity. **Conclusion**: Ki-ras gene mutation analysis in bile had a higher sensitivity in making a diagnosis of malignant stricture when compared with either brush or bile fluid cytology. However the application of this molecular markers in clinical practice is limited by the costy procedure, the need to improve the sensitivity and specificity of this marker by the combination of several markers, use of quanitative analysis to define threshold values for malignancy diagnosis. [Nature and Science. 2009;7(5):19-28]. (ISSN: 1545-0740).

Introduction

Early and accurate diagnosis of pancreaticobiliary malignancy offers the best chance of a surgical cure while avoiding unnecessary major surgery in patients with benign disease. However, despite many advances in biochemical testing, tumour markers, non-invasive imaging techniques, endoscopic retrograde cholangiopancreatography (ERCP), and various tissue sampling techniques, the nature of a stricture may remain unclear **Khalid et al., 2004.**

Although cholangiographic features at endoscopic retrograde cholangiopancreatography (ERCP) may be characteristic for malignant or benign disease, the diagnosis of malignant biliary strictures still rests on the identification of tumor cells obtained using various methods, including open biopsy, ultrasound or computed tomography guided fine needle aspiration or core biopsy, endoscopic forceps biopsy, endoscopic brush cytology, and bile aspiration cytology **Fogel & Sherman, 1999 & Ponchon ,2000.** Biliary and pancreatic duct lesions are not always readily accessible to biopsy, and cytological techniques have become the initial diagnostic modality in many cases. The reported sensitivities of bile or pancreatic juice cytology or brush cytology are highly variable but remain relatively low ranging from 6% (bile duct) to 85% (pancreatic duct)**Van laethem et al., 1998.** Cytology performed directly on bile allows detection of malignant cells in

only 6-26% of cancer cases **Foutch, 1994.**

Since mutations involved in neoplastic progression may be able to serve as markers for the presence of small numbers of neoplastic cells that would otherwise escape detection in diagnostic assays **Dillon et al., 2000**; they can improve the sensitivity of standard histopathological evaluation. In this aspect Ki-ras codon 12 mutations represents one of the earliest genetic changes in the development of pancreatic cancer **Almoguera et al., 1988**. Such point mutations mainly reside in the first two nucleotides of codon 12, making their detection by polymerase chain reaction (PCR) feasible Amplification of DNA from bile specimens is also possible, revealing point mutations associated with malignant strictures of the main bile duct **Dillon et al., 2000.**

The frequency of Ki-ras codon 12 mutation in pancreatic cancer was estimated at 75% to 95% when investigating histopathological specimen **Trumper et al., 2002**. There remains debate as to the exact frequency in bile duct cancer **Motojima et al., 1991, Tsuda et al., 1992** and thus in malignant stricture as an entity caused by a variety of disorders **Stewart et al., 2001**. The potential use of this molecular technique in bile samples for confirming the presence or absence of malignancy in patients with biliary stricture generated much interest and enthusiasm

This study aimed to evaluate the diagnostic value of detecting the Ki-ras gene mutation in bile samples compared to bile fluid cytology and brush cytology to differentiate between benign and malignant biliary strictures in a group of Egyptian patients.

Patients and Methods

1. Patients

This study was conducted on 40 patients with obstructive jaundice due to biliary strictures, referred to the ERCP unit at the Department of Gastroenterology and Hepatology, Ain Shams University Hospital. For each patient a definite diagnosis of the nature of the stricture was established by histological confirmation in tissue samples during ERCP or subsequent surgery or by adequate follow up of at least six months by imaging methods as CT scan in combination with the clinical disease course. None of the patients diagnosed to have benign stricture showed evidence of a worsening status or the presence of a tumour mass at follow-up CT.

Group I**:** Twenty five patients with malignant biliary strictures. They were 15 patients with pancreatic adenocarcinoma, 8 patients with ampullary carcinoma, 1 with cholangiocarcinoma and 1 with gall bladder cancer.

Group II: Fifteen patients with benign biliary strictures. They were 3 patients with primary sclerosing cholangitis, 4 patients with postoperative biliary stricture and 8 patients with postinflammatory stricture associated with gallstones and chronic pancreatitis.

All patients enrolled in the study had to give their written consent after sufficient explanation of the research and procedures to be done.

2. Sampling technique/ ERCP

Under conscious sedation using midazolam 5 mg & pethidine 25-50 mg, ERCP was performed using Pentax EPM-3500. The scope is side-viewing so that the papilla can be seen "face-on" and cannulated with a 1.7 mm teflon catheter through a scope conduit.

Bile fluid (12 ml) was aspirated from the common bile duct using a 20 ml dry syringe applied to a Wilson-Cook catheter. 10 ml of he aspirated bile fluid was then flushed into a 15 ml sterile BD Vacutainer, and then transported to the histopathology laboratory within an hour for bile fluid cytology **Jin et al., 1999**.

Another 2 ml of bile was collected in sterile test tubes then centrifuged at 12000 rpm for 15 minutes in a cooler centrifuge (Beckman GS- 15R centrifuge). The pellet was washed twice with phosphate buffered saline and subsequently stored at -80 °C until used for DNA extraction **Sambrook & Russel, 2001**. Then filling of both the pancreatic duct and the bile duct system was attempted. The contrast medium used was Urovideo 75% (Amidotrizoic acid), introduced under low pressure by hand injection under fluoroscopic control until entire filling was reached for radiographic documentation

The following findings were assessed during cholangiography:

1 The level of the biliary stricture: proximal, middle or distal

2 The pattern of the stricture: smooth tapering or abrupt cut-off

3 The presence of shouldering

4 The presence of mucosal irregularity

5 The presence of filling defects

Then a wire guided cytology brush system (Wilson-Cook Medical, Inc., Winston-Salem, N.C.) was used. The brush was opened into the lumen above the stricture and moved back and forth across the stricture, approximately 10 to 15 times, in a to-and-fro fashion. The brush was pulled just into the tip of the catheter, and the catheter withdrawn. After the brushing was performed, the brush was smeared on 4 slides. The slides were fixed immediately in 95% alcohol.

If possible Malleable forceps (Olympus America, Inc., Melville, N.Y.) was used to obtain tissue from the distal rim of the stricture or from ampullary mass. 2-4 bites were taken and fixed in 10% formalin for standard histopathological examination.

3. Histopathological examination

Bile fluid cytology and brush cytology slides were stained by Papanicolaou stain. The cytopathological criteria for malignancy included nuclear enlargement, pleomorphism (minimum of 3-4 fold variation in nuclear size), elevated nuclear/ cytoplasmic ratio, nuclear membrane irregularity and coarse Chromatin, loss of honeycomb arrangement **Solcia et al., , 1997** , **Jin et al., 1999**. Those showing minimal features of the above criteria not satisfactory for malignancy diagnosis (low grade atypia) were not considered in sensitivity and specificity calculation of the results.

4. Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) for Detection of Ki-ras Gene Point Mutation

A. DNA Preparation/Extraction:

Genomic DNA from the bile fluid pellets was extracted using the QIAamp DNA Mini Kit (QIAGEN Inc. Valencia, USA) according to manufacturer's instructions. To avoid DNA contamination, each of the samples was processed independently.

Measurements of DNA concentration were done by using Du series 640 spectrophotometer (Beckman Inc, USA).

B. DNA Amplification by Polymerase Chain Reaction (PCR):

The primers used (Metabion international AG, Deutschland) to detect the Ki-ras gene, were as follows:

- Primer (A) 5'-ACTGAATATAAACTTGTGGTAGTTGGACCT-3' (concentration: 100 Mm, molecular weight: 9260, number of bases: 30).
- Primer (B): 5'-TCAAAGAATGGTCCTGGACC-3' (concentration: 100 Mm, molecular weight: 6126, number of bases: 20)

- Primer (C): 5'-GCATATTAAAACAAGATTTAC-3' (concentration: 100 Mm, molecular weight: 6421, number of bases: 21)

To avoid false negatives, the presence of intact DNA was evaluated for each sample by control PCR using GAPDH. Sample that did not amplify with these primers were excluded from analysis. GAPDH was used as an internal control to monitor the integrity of the amplification process.

In a separate microfuge tube, the following components were prepared as a premix according to the number of samples, such that the total reaction volume was 50 µl.

Amplifications with Taq polymerase (Promega, Madison,W1) were performed in 100 µl reaction mixtures containing 2.5 units of Taq polymerase, 100 pmol of each primer, deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP) at 200 µM each, 1.5 mmol/L MgCl2, 60 mmol/L KCl, and 10 mmol/L Tris-HCl (pH 8.8). The reaction mixtures were subjected to amplification using a Thermal cycler (Biometra, Uno II Thermoblock), each cycle comprising 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 0.5 minutes. A negative (no DNA) control was run with each PCR analysis. The first PCR comprised 30 cycles followed by Mval digestion and a second PCR of 40 cycles.

C. Restriction Fragment Length Polymorphism Analysis (RFLP

Restriction enzyme digestion with Mval (Metabion international AG) was performed after each step of PCR in reaction buffer containing 16 µl of PCR product, 2 µl of the enzyme and 2 µl of the buffer. Mutation band was confirmed by electrophoresis of the second digested sample on 4% agarose gel and eluted.

PCR with primers A and B gave rise to a 157 base pair fragment containing two Mval restriction sites in case of normal codon 12, and just one site in case of presence of mutation in its first two bases. Therefore, wild type fragments cleaved to yield 114 base pairs, whereas mutant fragments yielded 143 base pairs.

The components used in polymerase chain reaction for K-*ras* gene detection were: Distilled water (30.75 µl), 10x reaction buffer (5µl) Primer 1 (sense) (5µl), Primer 2 (anti-sense) (5µl), dNTPs (10 Mm) (1µl), MgCl2 (25Mm) (3µl), Taq polymerase (5µ/µl) (0.25µl)

5. Statistical Analysis of the Results

Data were collected, revised then analyzed statistically using SPSS statistical package version 13. The following tests were applied: Mean value, Standard deviation, t- student test for independent sample means, Chi-square test

Sensitivity , Specificity , Positive predictive value (PPV) and Negative predictive value (NPV) were calculated as following: (Sensitivity = true positive/ true positive+ false negative, Specificity= true negative/ true negative+ false positive, Positive predictive value (PPV) = true positive/ true positive+ false positive & Negative predictive value (NPV) = true negative/ true negative+ false negative)

Significance was assumed when $P< 0.05$, high significance when $P<0.01$

Results

Ki-ras codon 12 analysis was successfully performed in 40 patients with obstructive jaundice due to biliary stricture [25 patients with malignant stricture and 15 patients with benign stricture], some demographic and laboratory data are shown in table (1) , Some ERCP findings are shown in table (2).

Table (1): Comparison between both studied groups as regard age, BMI, the serum liver enzymes and bilirubin.

variables	Benign n=15	Malignant n=25	t-value	p-value	significance
Age	45.8±17.9	60.2±10.1	3.257	<0.01	HS
BMI	26.4± 2.2	23.2± 1.6	-5.075	<0.01	HS
ALT (u/ml)	113.2□ 35.3	62.6 □28.5	-1.818	≥0.05	HS
AST (u/ml)	96.2 □71.6	90.1□ 51.2	-0.27	≥0.05	HS
ALP (u/ml)	230.8□ 130.4	493.8□ 206.2	4.424	<0.01	HS
γ-GT (u/ml)	117.0□ 90.9	239.0□ 123.0	3.329	<0.01	HS
Total bilirubin (mg/dl)	7.0□ 6.0	13.3□ 7.39	2.75	<0.01	HS
Direct bilrubin (mg/dl)	4.0□ 3.2	8.4□ 3.9	3.603	<0.01	HS

Table (2): Comparison between both studied groups as regard the radiological findings at ERCP.

sinificance	p-value	x2-value	Malignant	Benign	radiological findings
			n=25	n=15	
S	<0.05	4.235	6 (24.0%)	0 (0%)	Abrupt cut-off
S	<0.05	4.235	6 (24.0%)	0 (0%)	Mucosal irregularity
S	<0.05	4.952	12 (48.0%)	2 (13.3%)	Shouldering
H.S	<0.01	13.811	5 (20%)	12 (80.0%)	Smooth tapering

Overall 13 patients (52%) in malignant group had mutant Ki-ras gene and 2 patients (13.3%) in benign group (both with postinflammatory biliary stricture) were positive for Ki-ras mutation (table 3).
Distribution of Ki ras mutation in the malignant group is shown in table (4).

Table (3): Comparison between both studied groups as regard the presence of Ki-ras mutation by PCR.

Benign n=15	Malignant n=25	Ki-ras gene mutation
13 (86.7%)	12 (48.0%)	negative
2 (13.3%)	13 (52.0%)	positive
4.444	4.444	x^2 value
<0.05	<0.05	p value
Significant	Significant	Significance

Table (4): Distribution of Ki-ras mutation in malignant biliary strictures.

Malignancy	Ki-ras mutation		total
	negative	positive	n=25
Cancer pancreas	8 (53.3%)	7 (46.7%)	15
Ampullary cancer	2 (25%)	6 (75%)	8
Cholangiocarcinoma	1 (100.0%)	0 (0.0%)	1
Gall bladder cancer	1 (100.0%)	0 (0.0%)	1
x^2 value	0.746		
p value	≥0.05		
Significance	Not Significant		

Standard histopathological examination of bile fluid cytology and brush cytology was negative for malignancy in all patients in the benign group. In the malignant group only two patients were positive for malignancy in brush cytology, two patients show dysplasia described as few atypical cellular feature not satisfactory to diagnose malignancy (1 in bile fluid cytology & 1 in brush cytology) as shown in table 5

Table (5): Data of bile fluid cytology& brush cytology and Ki-ras mutation in the malignant group.

	Bile fluid cytology	Brush cytology	Ki-ras
	n=25	n=25	n=25
negative	24 (96%)	22 (88.0%)	12 (48.0%)
atypical	1 (4%)	1 (4%)	//
positive	0 (0%)	2 (8.0%)	13 (52.0%)
P value	<0.01		
significance	Highly significant		

For dignosis of malignancy sensitivity, specificity, PPV, NPV of bile fluid cytology, brush cytology and Ki ras gene mutation analysis are shown in table 6.

Table (6): Comparison between brush cytology and Ki-ras mutation in the malignant group as regards the sensitivity, specificity, positive predictive value and negative predictive values.

test	Bile fluid cytology	Brush cytology	Ki-ras mutation
sensitivity	0%	8.00%	52.00%
specificity	100%	100.00%	86.60%
PPV	0%	100.00%	86.60%
NPV	38.50%	40.50%	52.00%

Discussion

The diagnosis of malignant biliary strictures rests on the identification of tumor cells obtained using various methods; of which brush cytology performed at endoscopic retrograde cholangio-pancreatography (ERCP) has become a preferred method as it has a low complication rate and allows sampling from most sites within the pancreatic and biliary duct systems however it has modest sensitivity **Stewart et al., 2001**. It was hoped that the combination of standard diagnostic procedures & novel molecular tests designed to detect presumably tumour specific markers with a high sensitivity would greatly improved the diagnostic accuracy of biliary stricture **Trumper et al., 2002** . Ki-ras oncogene mutation is thought to be one of the earliest genetic changes in pancreatic cancer development **Almoguera et al., 1988.** It appears to be of biological significance in the complex process of cell transformation and has been described in several human carcinomas as well as in biliary tract cancers **Hruban et al, 1997**

In this study we compared the ability of Ki-ras codon 12 mutation analysis in bile fluid to diagnose malignancy compared to histopathological examination of brush cytology and bile fluid cytology.

Although brush cytology showed specificity of 100% for malignancy detection very low sensitivity (8%) was encountered. Data of previous studies are all in agreement concerning specificty of around 100%. The sensitivity rates reported previously are highly variable and range between 30% **Singh et al., 2003, Fogel et al.,2006** and 68% **Govil et al., 2002.** In a large series of 312 consecutive patients with extrahepatic bile duct stenosis the sensitivity of brush cytology for malignancy detection was not higher than 36% **Sturm et al., 1999**.

There are several possible explanations for the limited sensitivity of brush cytology in assessing pancreatic and biliary carcinomas. A low cellular yield is often the limiting factor in making a diagnosis of malignancy; in a recent study increasing brush size and bristle stiffness does not increase detection rates **Fogel et al., 2006**. In addition sampling errors might occur when tumours at these sites show a predominantly submucosal spread, with limited or absent surface epithelial abnormality. **Kurzawinski et al., 1993** & **Foutch, 1994** Similarly, strictures might be caused by external compression without directly involving the ductal epithelium that is why in general, results of brush cytology for biliary strictures induced by pancreatic malignancies have proved to be inferior (on average 46%) to those observed for biliary malignancies (on average 68%). **Glasbrenneret al., 1999**.

In addition some of these wide variations in sensitivity of brush cytology in reports arise from inconsistencies in the criteria used for classification of cells on cytological slides. These inconsistencies mainly arise in the categorization of lesions not fulfilling all criteria of malignancy. Classification of such cells has been termed to be a "cytological grey zone" and includes categories such as atypical, dysplasia (low and high grade), and suspicious **Selvaggi, 2004**. The morphological criteria used for this classification show significant overlap in various studies, and some authors even include suspicious or atypical lesions in the calculation of sensitivity. Thus comparison of the sensitivity and specificity rates obtained in various studies are hampered by the inconsistencies in the definition of cytological criteria in this "cytological grey zone" **Gress, 2004**

Concerning bile fluid cytology, all patients with benign strictures and 96% of those with malignant disease had a normal bile fluid cytology. Only one patient (4%) with malignancy had low grade atypia. It is reported in many studies that bile cytology is less sensitive than brush cytology **Kurzawinski et al.,1993 & Savader et al., 1998.** as in addition to the previously described factors limiting sensitivity of cytological examination of biliary malignancy in general, for bile fluid cytology a large volume of bile fluid may be needed to yield a few malignant cells.

In the present study Ki-ras gene mutation had a sensitivity of 52% and a specificity of 86.6% in diagnosing malignancy. This means that, according to our results, Ki-ras mutation had a higher sensitivity but a lower specificity in diagnosing a malignant biliary stricture.

A study by **Dillon et al., 2000** revealed that Ki-ras gene mutation had a sensitivity and specificity of

33% and 79% respectively. In the study performed by **Saurin et al., 2000**, the reported sensitivity, specificity and positive predictive values of Ki-ras mutation analysis in bile fluid samples of cases with malignant strictures were 17%, 96% and 94% respectively. Also, **Trumper et al., 2002** showed that Ki-ras mutation had a relatively low sensitivity of 38.1% but a high specificity of 90.5%. On the other hand **Van Laethem et al., 1998** compared Ki-ras mutation with brush cytology in 142 patients with malignant biliary strictures. The study reported a sensitivity of 25% versus 42% and a specificity of 100% versus 100% for Ki-ras mutation and brush cytology respectively.

The differences between the studies as regards the prevalence of Ki-ras mutations in malignant biliary strictures could be attributed to the different proportions of patients with malignancy enrolled in each study with different prevelance of the mutation which is reported in the literature to vary widely between 0% and 100 % **Gress, 2004**. According to **Saurin et al., 2000**, the prevalence of Ki-ras mutation in tissue samples obtained directly from the tumour was as follows: 90% in pancreatic adenocarcinoma, 77% in cholangiocarcinoma and 55% in gall bladder cancer and all these values are certainly higher than that detected in bile fluid cytology or brush cytology. The location of the biliary tumour (proximal or distal bile duct, intrahepatic bile ducts, gall bladder), racial and geographic variation, as well as the methods used for mutation detection have been assumed to cause these differences in the incidence of K-ras codon 12 mutations **Gress, 2004**.

The sensitivity of Ki-*ras* mutation analysis is however limited by the prevalence of these mutations in the tumour type under investigation, and is dependent on the presence of a sufficient amount of tumour cells in the biological sample used for the analysis. The specificity of Ki-ras PCR analysis is limited by the possible occurance of the mutation in non- malignant diseases of the pancreatic duct. **Furuya et al., 1997** showed that up to 37% of patients with chronic pancreatits showed mutation of the Ki-ras gene.

According to our study, the Ki-ras gene mutation was detected in the bile samples in 6 out of 8 (75%) cases of ampullary cancer. Contrary to our findings, **Ito et al., 1998 & Motojima et al., 1991** postulated that Ki-ras analysis was useful in differentiating true pancreatic from ampullary malignancy which are clinically likely to be confused in several cases as the later has no Ki-ras mutation. Although the number of ampullary cancer patients in our study was small, our findings at least highlighting the inappropriate use of Ki-ras mutation in this aspect in Egyptian patients till more data are available.

In conclusion, our study showed that Ki-ras gene mutation analysis in bile had a higher sensitivity in making a diagnosis of malignant stricture when compared with either brush or bile fluid cytology. However the application of this molecular markers in clinical practice is limited by the costy procedure, the need to improve the sensitivity and specificity of this marker by the combination of several markers, use of quanitative analysis to define threshold values for malignancy diagnosis as number of mutant cells will be much lower in inflammatory than in malignant cells.

Aknowledgement: The authors are grateful to many colleagues in Pathology department- Ain Shams University for their contribution to this study.

References

Almoguera C , Shibata D, Forrester K, et al., 1988: Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. Cell; 53: 549–55.

Dillon DA, **Johnson CC**, **Topazian MD et al., 2000:** The utility of Ki-ras mutation analysis in the cytologic diagnosis of pancreatobiliary neoplasma. *Cancer J*; 5:294-301.

Fogel EL and Sherman S, 1999: How to improve the accuracy of diagnosis of malignant biliary strictures. *Endoscopy*; 31:712-17.

Fogel EL, **deBellis M**, **McHenry L et al.,2006:** Effectiveness of a new long cytology brush in the evaluation of malignant biliary obstruction: a prospective study. Gastrointest Endosc.;63(1):71-7

Foutch PG, 1994: Diagnosis of cancer by cytologic methods performed during ERCP. Gastrointest Endosc; 40: 249–52.

Furuya N, Kawa S, Akamatsu T et al., 1997: long term follow up of patients with chronic pancreatitis and k-ras gene mutation detected in pancreatic juice. Gastroenterology 113: 593-8.

Glasbrenner B , Arden M, Boeck W et al., 1999: Prospective evaluation of brush cytology of biliary strictures during endoscopic retrograde cholangiopancreatography. Endoscopy ;31: 712–17.

Govil H, Reddy V, Kluskens L et al. 2002: Brush cytology of the biliary tract: retrospective study of 278 cases with histopathologic correlation. Diagn Cytopathol; 26(5):273-7.

Gress TM , 2004: Molecular diagnosis of pancreatobiliary malignancies in brush cytologies of biliary strictures. Gut ;53:1727-29

Hruban RH, Sturm PD, Slebos RJ, et al., 1997: Can Ki-ras codon 12 mutations be used to distinguish benign bile duct proliferations from metastases in the liver? A molecular analysis of 101 liver lesions from 93 patients. American Journal of Pathology; 151: 943-49

Ito R, Tamura K, Ashida H, et al., 1998: Usefulness of K-ras gene mutation at codon 12 in bile for diagnosing biliary strictures. Int J Oncol; 12(5):1019-23.

Jin YH, Kim SH & Park CK, 1999: Diagnostic criteria for malignancy in bile cytology and its usefulness. J Korean Med Sci;14: 643-7.

Khalid A, Pal R, Sasatomi E, et al., 2004: Use of microsatellite marker loss of heterozygosity in accurate diagnosis of pancreaticobiliary malignancy from brush cytology samples. Gut; 53(12):1860-5.

Kurzawinski T, Deery A, Davidson BR et al., 1993. Diagnostic value of cytology for biliary stricture. Br J Surg; 80:414–21.

Motojima K , Tsunoda T, Kanematsu T, et al., 1991: Distinguishing pancreatic carcinoma from other periampullary carcinomas by analysis of mutations in the Kirsten-ras oncogene. Ann Surg; 214: 657–62.

Ponchon T, 2000: Diagnostic endoscopic retrograde cholangiopancreatography. *Endoscopy*; 32:200–8.

Sambrook J & Russel DW, 2001: Molecular cloning: A laboratory manual. 8 th edition Cold Spring Harbor Laboratory Press.

Saurin JC, Joly-Pharaboz MO, Pernas P, et al., 2000: Detection of Ki-ras gene point mutations in bile specimens for the differential diagnosis of malignant and benign biliary strictures. *Gut*; 47(3):357-61.

Savader SJ, Lynch FC, Radvany MG et al., 1998: Single-specimen bile cytology: a prospective study of 80 patients with obstructive jaundice. J Vasc Interv Radiol**;** 9(5):817-21.

Selvaggi SM, 2004: Biliary brushing cytology. Cytopathology; 15:74–9

Singh V, Bhasin S, Nain CK et al., 2003: Brush cytology in malignant biliary obstruction. Indian J Pathol Microbiol; 46(2):197-200.

Solcia E, Capella C, Kloppel G., 1997: Tumors of the exorcine pancreas. In: Tumors of the pancreas. P.145. Eds.: Solcia E, Capella C, Kloppel G. Armed Forces Institute of Pathology, Washington.

Stewart CJR, Mills PR, Carter R, et al., 2001: Brush cytology in the assessment of pancreatico–biliary strictures: a review of 406 cases. Journal of Clinical Pathology; *54*: 449-455.

Sturm PDJ, Rauws EAJ, Hruban RH, et al., 1999: Clinical value of K-ras codon 12 analysis and endobiliary brush cytology for the diagnosis of malignant extrahepatic bile duct stenosis. Clin Cancer Res; 5:629-35.

Trumper L, **Menges M**, **Daus H et al., 2002:** Low sensitivity of the ki-ras polymerase chain reaction for diagnosing pancreatic cancer from pancreatic juice and bile: a multicenter prospective trial. J Clin Oncol; 20(21):4331-7.

Tsuda H , Satarug S, Bhudhisawasdi V, et al., 1992: Cholangiocarcinomas in Japanese and Thai patients: difference in etiology and incidence of point mutation of the c-Ki-ras proto-oncogene. Mol Carcinog; 6: 266–9.

Van Laethem JL, Bourgeois V, Parma J, et al., 1998: Relative contribution of Ki-ras gene analysis and brush cytology during ERCP for the diagnosis of biliary and pancreatic diseases. Gastrointest Endosc; 47(6):479-85.

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Which can Attenuate Hepatotoxicity Induced By Pesticides Mixture Natural or Synthetic Phenolic Antioxidant

Azza Gawish [a] and Manal E.A. Elhalwagy [b]

[a] Department of animal, faculty of scince , cairo university , Giza, Egypt
[b] Department of mammalian Toxicology , Pesticides central Laboratory , Agriculture Research Center,Giza,12611,Egypt

imankam_2@yahoo.com

Abstract: The present study examined the efficiency of green tea polyphenols as an example for natural polyphenols and butylated hydroxytoulene as an example for artificial polyphenols, in counteracting some of biochemical and histological alternations induced by repeated intoxication (28 days) with mixture of well known pesticides, widely investigated separetly. 6 groups of rats were treated as follows G1(control), G2 (p-mix , consists of, 1/60LD_{50} chloropyrifos =2mg/Kg b.wt, 1/200 LD_{50} of fenitrothion =2.5 mg/kgm b.wt and 1/100 LD_{50} of lambada cyhalothrin =0.17 mg/kg b.wt), G3(GT=100mg/animal), G4(p-mix+GT), G5(BHT=10mg/kgb.wt), G6(P-mix+BHT). Blood samples were taken at, 14 and 28 days for further biochemical parameters. Histopathological studies were carried out in liver tissue at the end of the experiment. Significant inhibition in plasma cholinesterase (ChE), damage in liver was observed and confirmed with elevation of plasma alanine aminotransferase (ALT), aspertate aminotransferase (AST) as well as elevation in oxidative stress (OS) marker malodialdehyde (MDA), plasma glucose, total cholesterol ,triglycerides and decrease in total glutathione content(GSH). In addition to angiogenic changes in blood vessels of animals treated with P-mix. Natural polyphenols (GT) supplemented to intoxicated rats induced pronounced counteracting effect in MDA, Glucose, cholesterol and triglycerides as well as promising effect in ALT&AST and liver tissue architecture and induce antiangiogenic effect. However, artificial polyphenols (BHT) supplementation has counteracting effect in MDA and GSH but it work synergistically with the p-mix on the other parameters. [Nature and Science. 2009;7(5):29-44]. (ISSN: 1545-0740).

Key Words: fenitrothion, chlorpyrifos, lambada cyhalothrin, mixture, polyphenols, green tea, butylated hydroxytoulene, oxidative stress, liver damage markers, angiogenesis

Introduction

Human and animal exposure to chemicals is rarely limited to a single chemical. Individuals are exposed daily to a variety of chemicals in food, drink, cosmetics and indoor and outdoor pollutants. In recent years, various environmental problems have led to increase concern about potential toxicity from exposure to multiple chemicals, including pesticide residues detected in food or water (Yang et al., 1989). Large-scale application of pesticides to crops and forests may contribute to the presence of toxic substances in the environment (John and Prakash, 2003). Pesticides are grouped into classes of compounds that have similar chemical structures and modes of toxic action. The most famous pesticide class is the organophosphate insecticides (OPs) organophosphorus (OPs) compounds are widely used in agriculture for vegetables and fruits protection, medicine and industry. Overspray of OPs resulting in serious damage to non-target species(Storm et al.,2000). Residual amounts of organophosphate (OP) pesticides have been detected in the soil, water bodies, vegetables, grains and other foods products (poet et al., 2004). Chlorpyrifos (CPF) and Fenitrothion, O, O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate, are organophosphorous insecticides, are now widely used for controlling a wide range of insects and pests. It was known that Ops cause acute toxicity through irreversible inhibition of acetylcholinesterase (ChE, EC 3.1.1.7), the enzyme responsible for the breakdown of the neurotransmitter acetylcholine. It has been reported that OPs may induce oxidative stress in humans (Almedia et al., 1997& Vidyasagar et al., 2004) and animals (Poovala et al., 1999 & verma 2001) when acutely exposed. On the other hand, it is well known that inhibition of acetylcholinesterase activity that is located in erythrocyte membranes can be an indicator of chronic toxicity of OPs. (Tinoco & Halperine 1998). Many other insecticide families also exhibit neurological activity and causes neurological damage, but at different target site as pyrethroids. Lambda-cyhalothrin is a broad-spectrum pyrethroid insecticide used to control a wide range of insect pests in a variety of crops. Lambda -cyhalothrin is highly used in the cotton plantation and in vegetable production (Leistra et al., 2003). Pyrethroids are potent sodium and potassium channel blockers that

produce subtle change in the channel's function, causing repetitive neural discharge (Soderlund et al., 2002). The mechanism by which pesticides cause damage involves multiple reaction pathways (Khan, 2006). Several studies of varying duration of exposure with organophosphorus or pyrethroid pesticides have postulated a possible role for the generation of free radicals and induction of oxidative stress (Tuzmen et al., 2008). In addition, it has been shown in previous studies that there is a correlation between acetylcholinesterase inhibition and lipid peroxidation levels in erythrocytes following subchronic and chronic exposure to OPs. (Akhgari et al., 2003 & Ranjbar et al., 2002). Other systems that could be affected by pesticides intoxication are immune system (Neishabouri et al., 2004) pancreas, liver and biochemical changes (Kalender et al., 2005). Polyphenols are our largest external source of antioxidants and are found in the plant foods that we eat. Polyphenols are naturally occurring chemicals and are responsible for the brightly colored pigments of many fruits and vegetables. Polyphenols have a significant antioxidant quality, by helping to protect tissues against oxidative stress (free radicals), certain polyphenols work as preventative medicines for problems such as cardiovascular diseases, cancer, arthritis, and auto immune disorders. Some have also exhibited anti-inflammatory and hepatoprotective effects. Polyphenols are secondary metabolites of plants and are widely distributed in plant derived foods such as cereals, legumes, nuts, vegetables, fruits and beverages such as green or black tea.(Bravo 1998). Polyphenols have been recently recognized as functionally active molecules, possessing antioxidant, anticancer rand antimutagenic properties (Chung et al., 1997). Tea polyphenols are the most significant group of green tea components, especially the catechin group of the flavonols. The major tea catechins are epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG), tea polyphenols possess a variety of biological functions, including antioxidant, anti-inflammatory, anticancer effects (Higdon & Frei 2003). Supplementation with black tea extract protects against free radical mediated oxidative stresss in hepatocytes of animals with pesticide mixture induced liver injury (Khan, 2006).Recently, tea polyphenols have been shown to protect against liver injury in animals intoxicated with chlorpyrifos insecticide (Khan & Kour 2007). Supplementation with (60mg/ animal) green tea polyphenols, partially attenuate oxidative stress resulted from the toxic effect of fenitrothion insecticide, on the liver and kidney of rats (Elhalwagy et al., 2008). The antiangiogenic property of green tea could be happened through multiple independent processes that include effects on gene expression signal processing or enzyme activities (Sogar et al., 2008). Phenolic antioxidant Butaylated hydroxytoluene (BHT), a preservative widely found in food as a food additive. Butylated hydroxytoluene (BHT) is known to inhibit tumor formation due to several chemical carcinogens including aflatoxin B1 (AFB1). The mechanism of action of BHT against AFB1 carcinogenesis is by induction of liver glutathione (GSH) S-transferases. As a result, the formation of AFB1-DNA binding is effectively inhibited (Allameh, 1997). Butylated hydroxytoluene (BHT) decreased the multiplicity of intestinal tumors (Balansky et al., 1992). Oral administration of BHT to rats also resulted in enhanced in vivo levels of GSH in lens, retina and cornea. In addition, a significant in vivo increase in the levels of GST, GSH-peroxidase, GSH-reductase, gamma-glutamylcysteine synthetase, and glucose 6-phosphate dehydrogenase was observed in the lens, retina, and cornea of BHT-fed rats (Ahmad et al., 1992). Fenirtrothion, chlorpyrifos and lambada cyhalothrin are well-known pesticides widely investigated separatly, and their effects on different organisms have been previously reported in separate studies. For this reason these pesticides were considered to be good model substances, relevant from the environmental perspective. On the other hand, we selected this kind of compounds because they are used in many tones annually in agriculture and horticulture and they are significant especially in greenhouse-based production of vegetables and fruits , In the present study, we investigated whether tea polyphenols or phenolic antioxidant (BHT) alleviate toxicity induced from mixture of the previous pesticides in rats.

Material &Methods

Animals

Male albino rats *Rattus norvegicus* (3–4) month's age, weighing between 150–180 g were used. Animals were supplied by the breeding unit of the Egyptian Organization for the Biology and Vaccine Production, Egypt. The animals were housed in plastic cages, fed *ad libitum* and allowed to adjust to the new environment for two weeks before starting the experiment. The rats were housed at 23 ± 2 ^{o}C dark/light cycle.

Chemicals:

Chlotpyrifos: pyriban (chlorpyrifos 48%EC) (O, O – Diethyl-O (3, 5, 6-trichloro-2-pyridyl phosphorothioat) was supplied by El Help company for pesticide industry- Egypt.

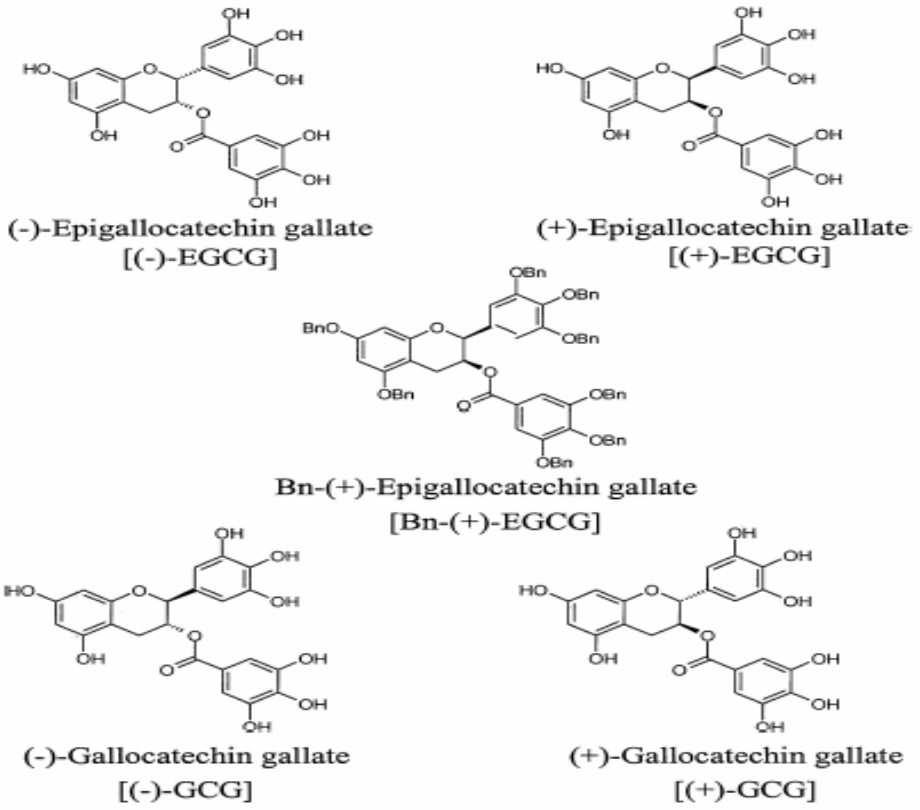
Fenitrothion: Sumithion (Fenitrothion 50% EC) (O,O-dimethyl O-4-nitro-m-tolyl phosphorothioate) was purchased from Kaffer Elzayat Co. for Insecticide Ind. Kaffr Elzayat, Egypt**.**

Lambada cyhalothrin: Karate (Lambda-cyhalothrin 2.5% EC) (cyano-3-phenoxybenzyl-3-(2-chloro-3, 3,3-trifluoro-1-propenyl)-2,2-dimethyl cyclopropanecarboxylate; is the most commonly and profusely used pyrethroid pesticide., was supplied by El Naser company for pesticide industry- Egypt.

Cocktail of combination of 3 types of pesticides were used in the present experiment

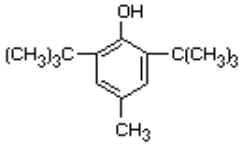
Antioxidant used:

Natural antioxidant: Green tea extract contains 98% polyphenols purchased from Hunan Changsha Yuanhang Biology Product Co., Ltd, China.contain a mixture of polyphenolic structures



Synthetic antioxidant:

Butylated hydroxytuleune (2:6-di-tert-butyl-p-cresol; 4-methyl-2:6-ditertiary-butylphenol) was purchased from Sigma Chemical Company (St. Louis, MO, USA).With chemical formula



Experimental Design:

All animals were treated according to the standard procedures laid down by OECD guidelines 407 (1992) repeated dose 28 days oral toxicity study in rodents. Animals were randomly divided into six experimental groups, five animals each as follows:

Group I (control): each animal in this group was given distilled water (1ml/animal) by gastric intubation every day for 28 days.

Group II (P-mix): rats were orally treated via gastric intubation with mixture of pesticides cocktail contain (1/60LD_{50} chloropyrifos =2mg/Kg b.wt, 1/200 LD_{50} of fenitrothion =2.5 mg/kgm b.wt and 1/100 LD_{50} of lambada cyhalothrin =0.17 mg/kg b.wt) every day for 28 consecutive days.

Group III(GT): rats were orally supplemented with 100mg /animal green tea extract for 28 days and served as(+ve control for GT) .

Group IIII (P-mix + GT): rats were orally supplemented with 100mg green tea/animal 1 hour after intoxication with pesticides mixture.

Group VII (BHT): rats were orally supplemented with 10mg /Kgm bwt butylated hydroxyl toluene for 28 days and served as(+ve control for BHT).

Group VIII (P-mix + BHT): rats were orally supplemented with 10mg/kgmbw butylated hydroxyl toulene 1 hour after intoxication with pesticides mixture.

Sampling

Blood samples collected from the retro-orbital plexus vein according to Schermer (1967). on heparinized tubes at 14 days and 28 days of treatment periods. Plasma samples were separated by centrifugation of the blood samples at 3600 rpm for 15 min. Plasma samples were kept at -20 C^{o} for subsequent use. At the end of the experiment, animals were dissected and samples of the liver were excised for histopathological studies.

Biochemical assay

Total reduced glutathione (GSH) was determined in erythrocytes by the method of Beutler et al. (1963) based on the development of a yellow color when DTNB is added to the supernatant of the precipitated RBCs containing sulfhydryl groups. Malondialdehyde (MDA) occurs as a result of lipid peroxidation in plasma and was measured according to Ohkawa et al. (1979) after incubation at 95 _C with thiobarbituric acid in aerobic conditions (pH 3.4). The pink color produced by these reactions was measured spectrophotometrically at 532 nm to measure malonaldehyde (MDA) levels. Plasma cholinesterase (ChE) was assayed by the method of Ellman et al. (1961). Markers for liver damage were determined using the commercial diagnostic kit of Stanbio Co., Spain. Plasma transaminases (AST and ALT) activities were determined according to Reitman and Frankel (1957). Cholesterol level was determined by the method of Henry(1974).Triglycerides were measured by the method of Schettler and Nussel(1975)**.** Plasma glucose level were determined according to Trinder (1959) using the commercial diagnostic kit of stanbio Co., Spain.

Histopathological Studies

Histopathological examination was carried out according to Drury and Wallington (1980). The liver tissue was dissected and the tissue samples were fixed in 10% formalin solution for 14–18 h, passed in a series of graded ethanol and embedded in paraffin. Paraffin sections were cut with at 5 µm thickness and stained with hematoxylin and eosin for light microscopic examination. The sections were examined and photographed on an Olympus light microscope (Olympus BX51, Tokyo, Japan) with attachment photograph machine (Olympus C-5050, Olympus Optical Co. Ltd., Japan). Identification of blood vessel areas for angiogenesis study, blood vessel areas was calculated by measurements of tissue boundaries and the area of all the blood vessels in the field this takes place by drowing a line around the blood vessel in each field, in one hot spot area. This was repeated in 5 fields by using the interactive morphometry software of the system on total magnification of (x100). The results appeared automatically on monitor in the form of the area of each blood vessel in each field measured in (2 µm) with total count and the area of all blood vessels in all the fields. The mean, the standard deviation, the minimum area, and the maximum area were measured (Niedergethmann et al., 2002)

Results

Effect of GT or BHT on Cholinesterase (ChE)

Inhibition in plasma acetyl cholinesterase (ChE) enzyme was noticed in P-mix intoxicated group significant as compared to the control at P<0.05 all through the experimental periods. However, animals supplemented with green tea polyphenols per se have a significant increase in ChE activity, it failed to counteract the effect of intoxication with P-mix. On the other hand, supplementation with BHT can not reduce the effect of p-mix intoxication but it work synergistically with p- mix to inhibit plasma ChE and this can be predicted from the effect of BHT per se as depicted in (Fig1).

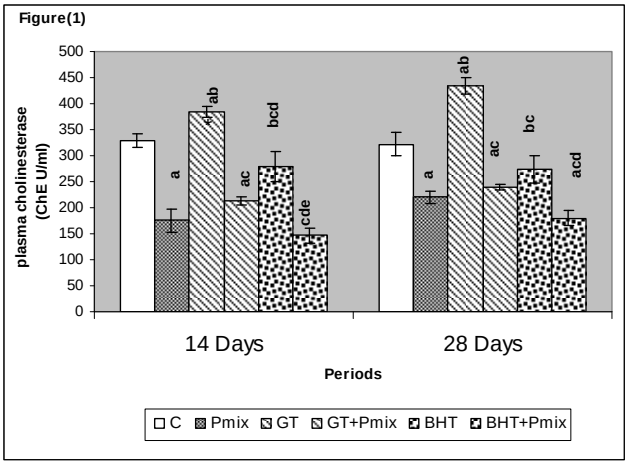


Figure (1): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) on acetylcholinesterase (ChE) in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE,[a] comparison of control and other groups($p < 0.05$), [b]comparison of pesticides mixture group and other groups($p < 0.05$),[c] comparison of green tea (+control) and other groups($p < 0.05$), [d] comparison of GT+ Pmix and other groups($p < 0.05$).

Effect of GT or BHT on Lipid peroxidation (MDA)

Intoxication with P-mix caused 1.5 fold increase in MDA level at 14& 28 days of treatment as compared to the control at P<0.05. Intoxicated rats supplemented with each of green tea polyphenols and BHT induced an early significant reduction in MDA level versus P-mix group to be reached nearly to the control level. However, slight reduction in MDA level was noticed at the end of the experiment significantly as compared to p-mix group (Fig 2).

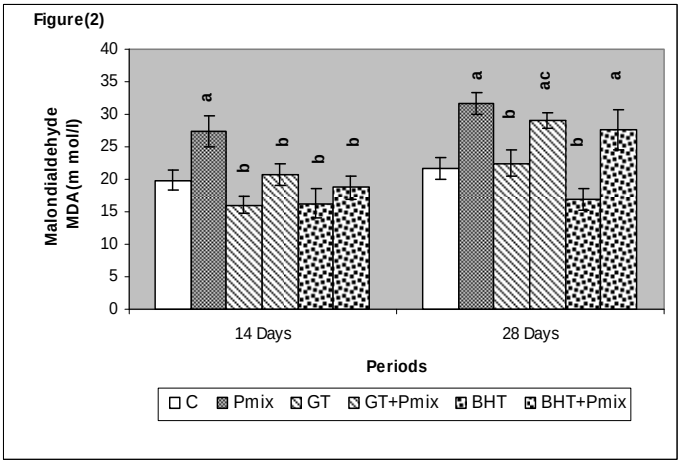


Figure (2): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) on malondialdehyde (MDA) lipid peroxidation biomarker in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups ($p < 0.05$), comparison of pesticides mixture group and other groups ($p < 0.05$),[c] comparison of green tea (+control) and other groups($p < 0.05$), [d] comparison of GT+ Pmix and other groups($p < 0.05$)

Effect of GT or BHT on Total glutathione (GSH)

A pronounced reduction in blood GSH level was recorded in P-mix group at the end of the treatment as compared to the control. Supplementation with each of natural and synthetic phenolic compounds to intoxicated animals slightly improve the level of GSH at 14th and 28th days, this improvement was pronounced in BHT supplemented group as compared to the other groups at P< 0.05(Fig 3).

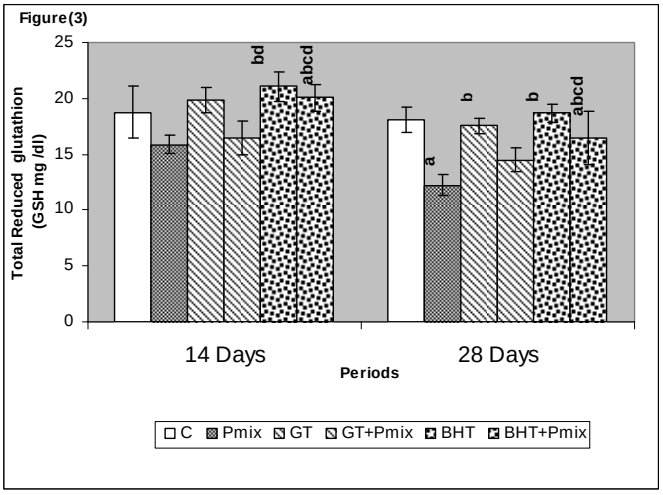


Figure (3): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) Total glutathione content (GSH) in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups(p <0.05),[b] comparison of pesticides mixture group and other groups(p <0.05),[c] comparison of green tea (+control) and other groups(p <0.05), [d] comparison of GT+ Pmix and other groups(p <0.05).

Effect of GT or BHT on Liver Damage Markers

ALT and AST levels were found to be significantly (p<0.05) raised in p-mix intoxicated group as compared to the control, the increment in each parameter was 2 and 3 folds respectively. While supplementation with green tea per se not significantly affects activity of ALT & AST, it failed to improve their activities when supplemented to P-mix intoxicated group. However, BHT supplemented group have pronounced elevation in enzymes activities all through the experimental periods, this elevation is significant versus control and the respective non supplemented groups at p< 0.05(Figure 4&5).

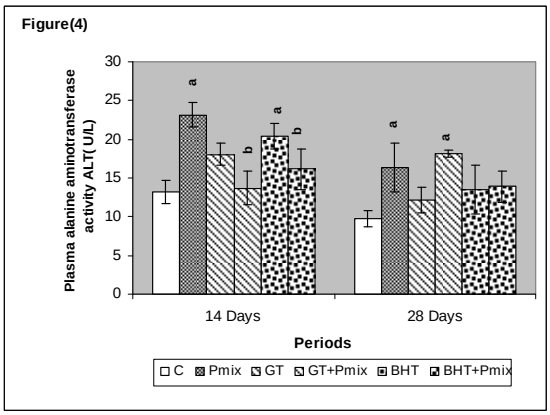


Figure (4): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) on Alaninamino Transferase (ALT) in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups (p<0.05), [b]comparison of pesticides mixture group and other groups (p<0.05),[c] comparison of green tea (+control) and other groups (p<0.05), [d] comparison of GT+ Pmix and other groups (p<0.05).

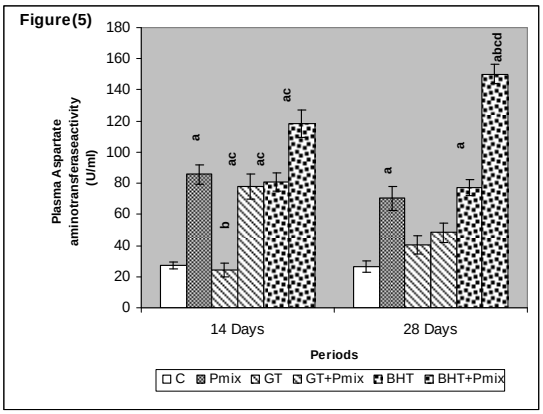


Figure (5): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) Aspartateamino Transferase (AST) in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups ($p<0.05$), [b]comparison of pesticides mixture group and other groups ($p<0.05$),[c] comparison of green tea (+control) and other groups ($p<0.05$), [d]comparison of GT+ Pmix and other groups ($p <0.05$).

Effect of GT or BHT on Plasma Glucose

A noticeable elevation in plasma glucose level was recorded in P-mix intoxicated animals, significant versus control at the end of 28th days of treatment. However administration of natural polyphenols (GT) attenuates this increment to be more or less near to the control level (Figure 6). On the other hands, intoxicated animals supplemented with artificial polyphenols (BHT) enhanced plasma glucose level to be elevated significantly versus control group and other treated groups, this effect must be attributed to the effect of BHT as demonstrated in group treated with BHT per se (Figure 6).

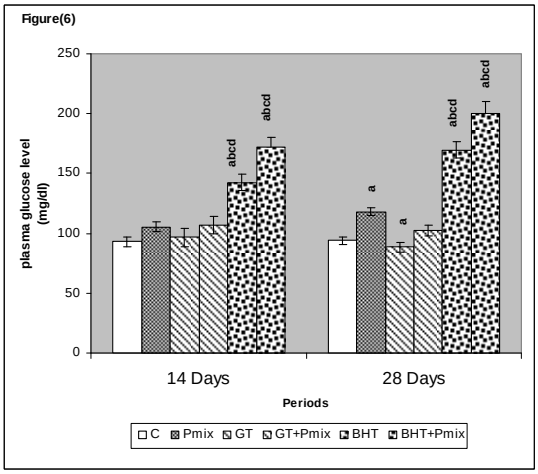


Figure (6): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) on Glucose Level in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups ($p<0.05$), [b]comparison of pesticides mixture group and other groups ($p<0.05$),[c] comparison of green tea (+control) and other groups ($p<0.05$), [d] comparison of GT+ Pmix and other groups ($p<0.05$).

Effect of GT or BHT on Cholesterol and Triglycerides

As depicted in (Figure 7 & 8) treatment with P-mix induced significant increase in each of plasma cholesterol and triglycerides versus control $p< 0.05$. Supplementation with natural polyphenols (GT) counteracts p-mix effects a remarkable significant reduction in cholesterol level was recorded at 28th day in a counterpart (83.83±9.11 to 40.45±7.10 mg/dl, respectively). However, level of triglycerides more or less

nearly reached to the control level. On the other hand, supplementation with BHT failed to reduce the toxicity effect of p-mix. Significant increase in cholesterol and triglycerides level was recorded versus control and the respective non supplemented group at $p<0.05$.

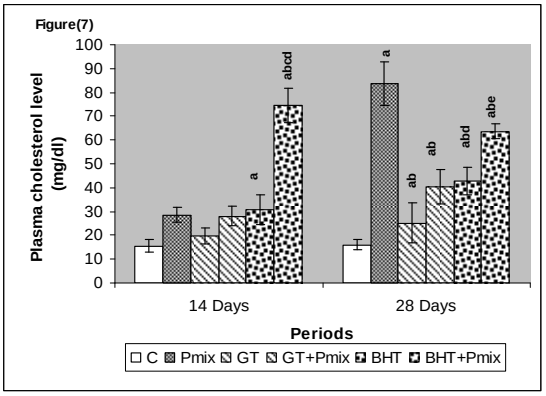


Figure (7): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) on total cholesterol in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups($p<0.05$), [b]comparison of pesticides mixture group and other groups($p<0.05$),[c] comparison of green tea (+control) and other groups($p<0.05$), [d] comparison of GT+ Pmix and other groups($p<0.05$).

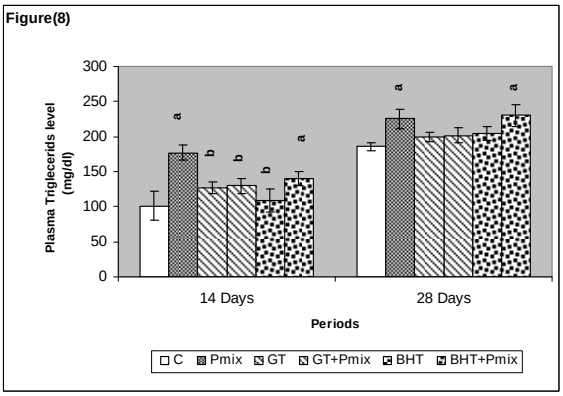


Figure (8): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) Triglecerides in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups($p<0.05$), [b]comparison of pesticides mixture group and other groups($p<0.05$), [c]comparison of green tea (+control) and other groups($p<0.05$), [d] comparison of GT+ Pmix and other groups($p<0.05$).

Histopathological results

(Fig.9a) showed normal liver architecture with the central vein and radiating cords of normal hepatocytes with central rounded nuclei. Normal blood sinusoids appeared between the liver cords Liver section of rats administered orally with pesticides mixture for 28 days showing fatty liver and no assembly of the liver cells, depletion of their outer membranes with pyknotic nuclei (Fig. 9b). Liver section of rats supplemented orally with 100mg green tea extract/animal for 28 days per se and served as +ve control showing nearly normal structure of liver cells besides some of the fatty contents (Fig.9c). On the other hand, liver section of rats supplemented orally with 100mg green tea extract/animal 1 hour after intoxication with pesticides mixture for 28 days showing some central nuclei with undefined shapes and irregular cells with depletion in their cytoplasm, but the liver architecture was preserved that may consider some improvement but it needs more time in GT polyphenols supplementation (Fig 9d). Liver section of rat supplemented orally with 10mg/kg.b.wt. Butylated hydroxyl toluene (BHT) for 28 days showing more or less hepatic cells suffering from granulation and empty areas of their cytoplasm and irregular nuclei (Fig 9e). Liver section of rats supplemented orally with 10mg/kg.b.wt. Butylated hydroxyl toluene (BHT) and

pesticides mixture for 28 days showing depletion in the cytoplasm of hepatic cells with irregular nuclei (Fig 9f).

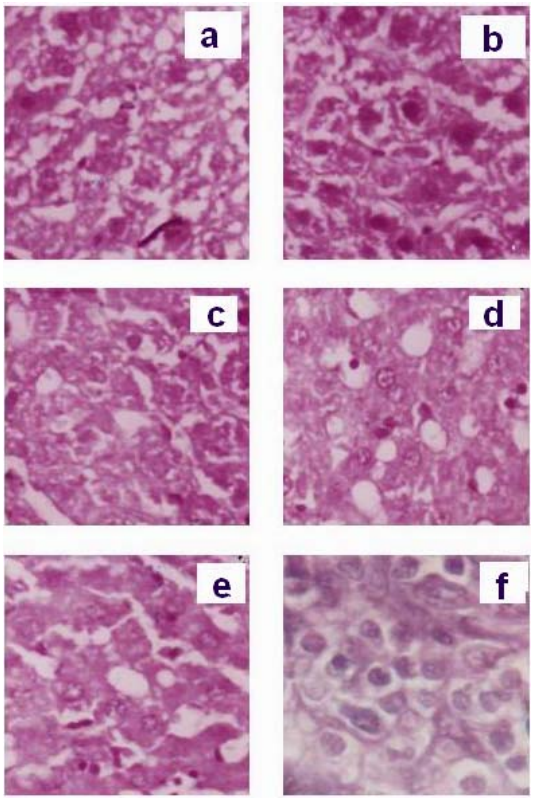


Fig. **(9):** Photomicrograph of the liver sections. Untreated control rat, showing a normal architecture of their hepatic cells (arrows) (**a**). Liver section of rat administered orally with pesticides mixture for 28 days showing fatty accumulation between the hepatic cells; depletion of the outer hepatic cell membrane with pyknosis of their nuclei (**b**) (arrows). Liver sections of rat supplemented with 100 mg green tea extract for 28 days showing normal of liver cells ©. Liver sections of rat supplemented with 100 mg green tea extract 1 hour after intoxication with the pesticide mixture for 28 days showing undefined nuclei and irregular cells with depletion of their nuclei (arrows) **(d**).Liver section of rat supplemented orally with 10mg/kg of butylated hyroxyl toluene (BHT) for 28 days showing more or less hepatic cells suffered from granulation and empty areas of their cytoplasm and irregular nuclei (arrows) **(e).** Liver sections of rat supplemented orally with 10mg/kg of BHT and intoxicated with the pesticide mixture for 28 days showing depletion of the cytoplasm of hepatic cells with irregular nuclei (arrows)**(f).** All photomicrographs stained with H & E 400X**.**

Immunohistological (Antiangiogenesis) results

As expressed in table 1 and fig. 10a which showed the liver sections of untreated rat, abundance blood vessels and hepatic cells immunolabelled with monoclonal antibody directed against the Factor-VIII-associated antigen. Liver section of rats administered orally with pesticides mixture for 28 days showing decrease in the invading blood vessels between hepatic cells. The results of measured areas of blood vessels in the hot spot areas showed decrease (35.9±6.88) in the blood vessel areas compared to control group (Table 1 and fig.10b). Liver section of rat supplemented orally with 100mg green tea extract/animal for 28 days showing nearly normal invading blood vessels and hepatic sinuses. The measured values showed significant increase in the values (141.99 ± 39.44), table 1 and fig10c. Liver section of rats supplemented orally with 100mg green tea extract/animal 1 hour after intoxication with pesticides mixture for 28 days showing an increase in the blood vessels. The measured values obtained non significant increase (79.28 ± 18.14) compared to the control group (Table 1 and fig.10d). Liver section of rat supplemented orally with 10mg/k.b.wt. butylated hydroxyl toluene (BHT) for 28 days showing that some enlargement in the blood vessels between hepatic cells. The values showed no significant increase (67.76 ± 12.06), table 1 and fig.10e. Liver section of rats supplemented orally with 10mg/k.b.wt. butylated hydroxyl toluene (BHT) and

pesticides mixture for 28 days showing some constrictions of blood vessels and hepatic sinuses. Significant increase was noticed in the measured blood vessels areas (61.65 ± 17.61) compared to control group (Table 1 and fig.10 f).

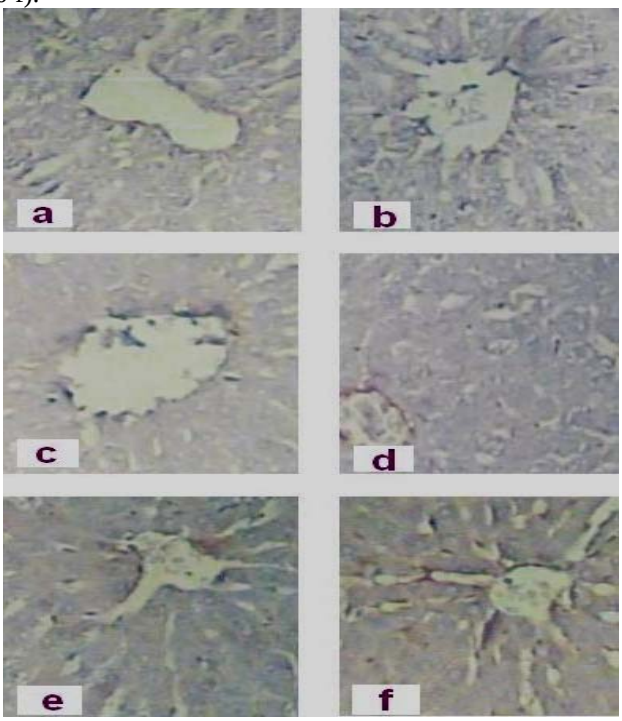


Fig (10): Photomicrograph of the liver section of untreated rat showing abundance blood vessels between the hepatic cells immunolabelled with monoclonal antibody against the factor – VIII associated antigen **(a)**. Liver section of rat administered orally with pesticide mixture for 28 days showing decrease in the invading hepatic vessels immunolabelled with monoclonal antibody against the factor – VIII associated antigen **(b).** Liver section of rat supplemented orally with 100mg/kg of green tea extract for 28 days showing nearly normal blood vessels and hepatic sinusoids immunolabelled with monoclonal antibody against the factor – VIII associated antigen **(c).** Liver section of rat supplemented orally with 100mg/kg of green tea extract 1 hour after intoxication with pesticide mixture for 28 days immunolabelled with monoclonal antibody against the factor – VIII associated antigen showing an increase in the blood vessels growing **(d).** Liver section of rat supplemented orally with 10mg/kg of butylated hydroxyl toluene for 28 days immunolabelled with monoclonal antibody against the factor – VIII associated antigen showing some enlargement of the diameters of the hepatic vessels **(e).** Liver section of rat supplemented orally with 10mg/kg of butylated hydroxyl toluene BHT 1 hour after intoxication of pesticide mixture for 28 days immunolabelled with monoclonal antibody against the factor – VIII associated antigen showing constrictions between the hepatic vessels **(f).**

Table (1): The Angiogenesis effects of Green Tea polyphenols and BHT polyphenols on the toxicity of pesticides mixture

Groups	control	P-mix	GT (+vecontrol)	Pmix +GT	BHT (+vecontrol)	P-mix +BHT
Mean + SE	55.8 + 7.42	35.92 + 6.88*	141.99 + 39.33*	79.28 + 18.14	67.76 + 12.06	61.65 + 17.61*
Max Area	120.8	100.72	553.07	293.86	261.43	317.91
Mini Area	9.8	12.02	14.7	17.56	17.59	11.74

Discussion

Several studies had been conducted to investigate the adverse effects induced as a result of individual exposure to different pesticides. Fenitrothion, lambada cyhalothrin and chlorpyrifos insecticides included in our examined mixture. They have been examined individually in previous studies by (El-Halwagy et al., 2008 , El-Demerdash 2007). Substantial information is available regarding their environmental and ecological impact , not much is known in regard to the mixture toxicity in mammalian system . The mechanism by which pesticide cause damage varied according the structure of the pesticides, the primary mechanism of action and most acutely life threaten effect of Ops insecticides are related to the accumulation of acetylcholine within the cholinergic synapses resulting from inhibition of acetyl cholinesterase by active oxon metabolites (Mileson et al., 1998). The main effects of pyrethroids are on sodium and chloride channels, pyrethroid modify the gating characteristics of voltage sensitive sodium channels to delay their closure (Bradberry et al., 2005). Increasing Na+ influx into synaptic terminals and creating a hypopolarized hyper irritable synaptic membrane, which in turn increases the release of the neurotransmitter acetylcholine (Bandettini et al., 1992 and Rao & Rao, 1993). Previous facts explain the remarkable inhibition in plasma ChE induced when animals treated with cocktail (Fn+CPF +lambada cyhalothrin) mixture , these results are coincide with that recorded by Latuszynska et al., 2001 and 2003; chlorpyrifos and cypermethrin administrated in a mixture strongly inhibited cholinesterase activity in plasma, this inhibition was associated with the effect of chlorpyrifos. An increase in the amount of dissolved oxygen and reactive oxygen species (ROS) in the blood and excessive generation of highly reactive oxidants results in tissue damage, called as oxidative stress. ROS are derived from a variety of sources, such as the xanthine oxidase system, activated neutrophils, the electron transport chain of mitochondria, and the arachidonic acid pathway. Since free radicals have very short half-lives, the clinical assessment of oxidative stress in vivo is based on the measurement of different stable oxidized products of modified lipids, proteins, carbohydrates and nucleic acids. Malonyldialdehyde (MDA), is one of the most widely used biomarkers of oxidative stress, is produced enzymatically by the breakdown of unstable hydro peroxides during per oxidation of unsaturated fatty acylmoietie (Roberts and Morrow 2000). Our results demonstrated a significant increase in plasma MDA level after intoxication with cocktaile of pesticides for 28 days. It is plausible to speculate from our results that (CPF, Fn and lambada cyhalothrin) treatment may result in peroxidation of polyunsaturated fatty acids , leading to degradiation of phospholipids and ultimately result in cellular deterioration (Tappel.1973), science coktaile of investigated pesticides are lipophelic substances, they may interacting with the cellular plasma membrane (Hazarika et al.,2003). Depletion in total glutathione content GSH resulted from intoxication with pesticides mixture and concurrent to the elevation of lipid peroxidation biomarker MDA. Reduced glutathione plays an important role in the detoxification of xenobiotic and antioxidation of reactive oxygen species and free radicals by oxidation of GSH to glutathione disulfide (GSSG) so increasing in oxidative stress accompanied by decline of GSH as reported by (Manna et al., 2005). The extent of liver damage appears to be considerable as evidenced by the increase in plasma levels of ALT & AST as shown in Figure (5&6) resulted from intoxication with cocktail of pesticides, these results are in content of the previous results recorded by (Elhalwagy et al.,2008, Khan,2006 and Muthuviveganandavel et al., 2008) oral intoxication with each of fenitrothion ,chlorpyrifos and cypermethrin ,respectively, induced elevation in ALT & AST activities as a result of liver tissues damage expressed by histological examination and run parallel with marked histological alterations were observed in the liver of rats treated with pesticide mixture for 28 days in our study in which tissue disorganized, cytoplasmic vacuolization (fatty degeneration), cellular necrosis and congestion of blood vessels. One of the characteristics of pesticides is induction of stress, stress is a response to every situations which threatening homeostasis and result in activation of hypothalamic pituitary adrenal (APA) axis and sympathetic autonomic nervous system, which consequently lead to hyperglycemia, (Mechanick, 2006). Stimulation of sympathetic nervous system during stress leads to enhanced release of catecholamines, glucagon and growth hormone which result in promotion of gluconeogenesis, glycogenolysis, insulin resistance and constitution of hyprerglycemia. Also, Ayub shah & Gupta (1997) & Husain et al., (1994) recorded an elevation in glucose level as a result of permethrin or deltamethrin intoxication mainwhile, (Rahimi and Abdollahi 2007) reported significant elevation in glucose level after OPs intoxication, these facts explain significant elevation in plasma glucose as a result of pesticides mixture intoxication recorded in the present study. Our data also, pin point the role of pesticides mixture on the elevation of the level of plasma total cholesterol and triglycerides, the increase in the level of serum cholesterol may be due to increase synthesis of serum cholesterol in the liver (Enan et al., 1987)

or may be attributed to an inhibition of lipase lipoproteins (Goldberg et al., 1982). Each of artificial or natural polyphenols supplementation to pesticides mixture intoxicated animals, failed to counteract the inhibition in plasma ChE induced by pesticides cocktail intoxication. Catechins one of green tea polyphenols reacts with peroxyl radicals in phospholipids bilayers via a single electron transfer followed by deprotonation prevent inhibition in AchE enzyme (Javanovic et al., 1996), these findings explain the significant increase in ChE enzyme activity in plasma of animals supplemented with green tea polyphenols and served as positive control. However, artificial polyphenols BHT works synergistically with the inhibitory effect of pesticides mixture intoxication on plasma ChE. It must be noted here that BHT per se has an inhibitory effect on plasma ChE as depicted in previous study reported by (Bilusic et al., 2008). With respect to the effect of polyphenols supplementation on lipid peroxidation biomarker (MDA) each of BHT & green tea polyphenols try to counteract the increase in MDA resulted from P-mix intoxication to be more or less near the control level. Green tea polyphenols do its effect on lipid per oxidation by enhancing the level of antioxidant directly of spare the endogenous pool of GSH from being exhausted by generated free radicals (Skrzydlewska et al., 2002& Kane 2006). A molecule of BHT is able to react with 2 peroxyl radicals to yield products that bare more stable (Black 2004) as well as it enhance the level of GSH (Ahmad et al.,1992). The previous findings run parallel with our findings in GSH results. green tea obtained ameliorating affect in the liver section of rats with some fatty degeneration. (Zhen et al., 2007) recorded that EGCG content of green tea arrested progression of hepatic fibrosis. (Feng et al., 2002) reported that tea flavines could prevent cellular DNA damage by inhibiting oxidative stress. Also, (Zhong et al., 2003), reported that polyphenols of green tea scavenge oxygen radicals and prevent activation of stellate cells minimizing liver fibrosis. green tea polyphenols needs more time to ameliorate the toxic effect of pesticides on ALT & AST (Elhalwagy et al., 2008) . Significant reducation in cholesterol and triglycerids were observed in green tea polyphenols supplemented groups, this obtained results are in consistant in previous studies (Matsumoto et al., 1998). In contrast, BHT failed to counteract the elevation in plasma cholesterol and induced triglycerides , these results are coincide with that obtained by (Faine et al.,2006) In spite of improvement on the oxidative stress parameters , an enhancement in the liver enzymes biomarkers were recorded in BHT polyphenols supplemented groups permeabilized the plasma and mitochondrial membranes to enzymes leakage accompanied by enhanced membrane fluidity in animals treated with BHT (Shertezer et al., 1991). BHT is an inducer of cytochrome P450 in the hepatocytes (Price et al., 2008), it was recorded that BHT was a multiple mechanisms especially cytotoxic effect through interactions with neutrophils membranes and the ROS scavenging effect (Kobeya et al., 2008). However, (Guarisco et al., 2008) showed that BHT is capable to induce oxidative and metabolic alterations similarly to some pathological disorders. Vessel formation occurs mainly through two sequential mechanisms (Carmeliet, 2000). De novo formation of blood vessels during embryonic development is called vasculogenesis. Mesoderm-derived stem cells (hemangioblasts) form aggregates (blood islands), and they develop into primitive hematopoietic and endothelial cells (angioblasts). Angioblasts differentiate and proliferate in situ to form a primitive network. On the other hand, the formation of new capillaries from preexisting vessels is
called angiogenesis. The principle mechanism of vessel formation in adults is angiogenesis.

As regards to the antiangiogenic results revealed nearly normal invading blood vessels and hepatic sinuses. (Jung et al., 2001) and (Zhang et al., 2006) recorded that EGCG of green tea inhibited the increase of VEGF expression, blocking its induction and leading to the antiangiogenesic effect of green tea. Other investigation by inhibiting metalloproteins and the vascular endothelial growth factor.

In conclusion, exposure to pesticides mixtures showed highly degeneration of hepatic cells and the antiangiogenic effect decrease in the invading blood vessels between hepatic cells as well as disturbance in different biochemical parameters. Supplementation with natural polyphenols as green tea showed some ameliorating effects but may be needs more time for the present investigated dose and another doses can be investigated . However, artificial polyphenols BHT increases the incidence of degeneration of hepatic cells. Although, there is an improvement in oxidative stress parameters and enhancement of GSH level.

References

1. Ahmad H, Sharma R, Mansour A, Awasthi YC.(1992): t-butylated hydroxytoluene enhances intracellular levels of glutathione and related enzymes of rat lens in vitro organ culture. Exp Eye Res. Jan; 54(1):41-8.

2. Akhgari M, Abdollahi M, Kebriaeezadeh A, Hosseini R, Sabzevari O.(2003): Biochemical evidence for free radical induced lipid peroxidation as a mechanism for subchronic toxicity of malathion in blood and liver of rats. Hum Exp Toxicol 22: 205- 11.
3. Allameh A.(1997): Comparison of the effect of low- and high-dose dietary butylated hydroxytolueneon microsome-mediated aflatoxin B1-DNA binding. Cancer Lett. 1997 Mar 19;114(1-2):217-20.
4. Almedia, M.G; Fanini F., Davino S, Aznar AE, Koch OR, Barros SBM. 1997: antioxidant parameters in rat liver after short-term exposure to hexacholorobenzene. Hum Exp Toxicol 16: 257- 61.
5. Ayub Shah MA, Gupta PK. (1997): Biochemico-toxicological study on permethrin a synthetic pyrethroid insecticde in rats .Indian J Toxicol;4:57-60.
6. Balansky R, Blagoeva P, Mircheva Z, Pozharisski K, de Flora S.(1992): Effect of metabolic inhibitors, methylxanthines, antioxidants, alkali metals, and corn oil on 1,2-dimethylhydrazine carcinogenicity in rats. Anticancer Res. May-Jun;12(3):933-40.
7. Bandettini, P.; Eells, J.; Holman, P. and prop, J. (1992): Pyrethroid insecticide induced alternation in mammalian synaptic membrane potential. J. Pharmacol. Exp. Ther., 262:1173-1181.
8. Beutler, E.; Duron, O.; Kelly, M.B. (1963): Improved method for the determination of blood glutathione, J. Lab. Clin. Med. 61: 882–888.
9. Bilusic,K.T.; Katalinic,V.; Uzelac,V.D.; Ljubenkov, I.; Krisko,A. Dejanovic,B.;Jukic,M.;Politeo,O.;Pifat,G. and Milos,M. (2008): Antioxidant and acetylcholinesterase inhibiting activity of aqueous tea infusion in vitro. Food Technol.Biotechnol.46(4) 368-375.
10. Black, H.S. (2004) : Reassessment of a Free Radical Theory of Cancer With Emphasis on Ultraviolet Carcinogenesis. Integrative Cancer Therapies. 3(4); pp. 279-293
11. Bradberry, S.M.; Cage, S.A.; Proudfoot, A.T. and Allister, V.J. (2005): Poisoning due to pyrethroids. Toxicol. Rev., 24(2):93-106.
12. Bravo,L.(1998) polyphenols: chemistry,dietary sources, metabolism and nutritional significance. Nutr.Rev.,56,317-333.
13. Carmeliet P. (2000) Mechanisms of angiogenesis and arteriogenesis. Nat Med.; 6: 389–95.
14. Chung,S.Y., Lee,M.J.,Chen,I. and Yang,G.y.(1997) Polyphenols as inhebitors of carcinogenesis. Environ.health Perspect.,105,971-976.
15. Drury, R.A.; Wallington, E.A.; Carleton,s (1980): Histological Techniques,fifth ed., Oxford University Press, London, New York, Toronto, pp. 241–242.
16. El-Demerdash, M.F;(2007): Lambda-cyhalothrin-induced changes in oxidative stress biomarkers in rabbit erythrocytes and alleviation effect of some antioxidants. Toxicology in vitro,21:392-397.
17. Elhalwagy,EAM; Darwish,NS; Zaher,EM.(2008): Prophylactic effect of green tea polyphenols against liver and kidney injury induced by fenitrothion insecticides.Pesti.Bio.Physio.91,81-89.
18. Ellman,G.L; Couriney, K.D.; Andres, V.J.R.; Featherstone, R.M.A (1961): new and rapid colorimetric determination of acetyl cholinesterase activity, Biochem. Pharmacol. 7: 88–95.
19. Enan; E, Berberian; I.G.,El fiky;S.;Elmasry,M.Enan;O.H. (1987): Effect of organophosphorus insecticides on some biochemical constituent in the nervous system and liver of rabbits. J. Environmen. Sci. Health B22: 149-70.
20. F.A. Davi, Co., Philadelphia, p. 42.
21. Faine, L.A.; Rodrinues,H.G.; Galhardi,C.M; Ebaid, G.M; Diniz, Y.S; Fernandes, A.A;Novelli,E.L (2006): Butylated hydroxytoluene (BHT) induced oxidative stress : effects on serum lipids and cardiac energy metabolism in rats. Experimental and Toxicologic pathology. Vol,57(3) 221-226.
22. Feng, Q.; Torii, Y.; Uchida, K.; Nakamura, Y. Hara, Y. and Osawa, T. (2002); Black tea polyphenols, tea flavines, prevent cellular damage by inhibiting oxidative stress and suppressing cytochrome p450 in cell cultured. J. Agric. Food. Chem. 50(1): 213-220
23. Goldberg, I.J.; Paterniti, J.R.; Ginsberg, H.N.; Lindgren, F. T. and Brown, W.V. (1982): Lipoprotein metabolism during acute inhibition of hepatic triglyceride lipase in the Cynomolgus monkey. J. Clin. Invest., 70:1184-1192.

24. Guarisco, J. A.; Hall, J.O.; coloumbe, R.A.; (2008): Mechanisms of BHTchemopreention of aflatoxicosis inhibition B1 metaboliswm. Toxicol. Appl. Pharmacol. 227(3): 339-46.
25. Hazarika,A.; Sarka,S.; Kataria,N.; Malik, M.K. (2003): Influence of malathion pretreatment on the toxicity of anilofos in male rats: a biochemical interaction study, Toxicology 185 (2003) 1–8.
26. Henry, R.J.U.M. in: Z. AuX (Ed.) (1974): Clinical Chemistry, Harper and Row, Publishers, New York, pp. 1440–1443.
27. Higdon J V,Frei B, (2003): Tea catechins and polyphenols:health effects, metabolism,and antioxidant functions.Crit Rev Food Sci Nutr.43:89-143.
28. Husain R, Husain R, Adhami VM , Seth PK. Behavioural (1994): neurochemical and neuromorphological effects of deltamethrin in adult rats. J Toxicol Environ Hlth 48:515-26.
29. Javanovic, S.V; Steenkn, S; Hara, Y.; Sinic, M.G. (1996): Reduction potential of flavonoid and model phenoxyl radicals. Which ring in flavonoids isresponsible for antioxidant activity? J. Chem. Soc. Perkin Trans. 2:2497–2504.
30. John, P.J., Prakash, A., 2003. Bioaccumulation of pesticides on some organs of freshwater catfish Mystus vitatus. Bull. Environ. Contam. Toxicol. 70, 1013– 1016.
31. Jung, Y.; Kim, M.S.; Shin, B.A.; Chay, K.O; Ellis, L. (2001): EGCG, a major component of green tea inhibits tumor growth by inhibiting VEGF induction of human colon carcinoma cells Br. J. Cancer. 84(6): 844-850.
32. Kabeya, L. M.; Kanashiro, A.; Azzolini, A.E. and Santos, A.C. (2008): Antioxidants activity and cytotoxicity as mediators of neutrophil chemiluminescence inhibition by BHT. Pharmazie. 63(1): 67-70.
33. Kalender, S.; Ogutcu, A. ; Uzunhisarcikli, M.; Acikgoz, F. Durak, D.; Ulusoy, Y.; Kalender, Y. (2005): Diazinon-induced hepatotoxicity and protective effect of vitamin E on some biochemical indices and ultrastructural changes, Toxicology 211 (2005) 197–206.
34. Khan, S.M. (2006): protective effect of black tea extract on the levels of lipid peroxidation and antioxidant enzymes in liver of mice with pesticide – induced liver injury. Cell biochem funct .24,327-332.
35. Khan,S.M.and Kour,G.(2007): Subacute oral toxicity of chlorpyriphos and protective effect of green tea extract Pesti.Bio.Physio.89,118 - 123.
36. Latuszynska, J.; Luty, S.; Raszewski, G.; Przebirowska, D.; and Tokarska, R. M. (2003): Neurotoxic Effect Of dermally-applied chlorpyrifos and cypermethrin. Reversibility of changes. Ann. Agric. Environ. Med., 10:197-201.
37. Latuszynska, J.; Luty, S.; Raszewski, G.; Tokarska, R.M.; Przebirowska, D.; Przylepa, E. and Haratym-Maj, A. (2001): Neurotoxic Effect Of dermally-applied chlorpyrifos and cypermethrin in wister rats. Ann. Agric. Environ. Med., 8:163-170.
38. Leistra, M., Zweers, A.J., Warinton, J.S., Crum, S.C.H., Hand, L.H., Beltman, W.H.J., Maund, S.J., 2003: Fate of the insecticide lambda-cyhalothrin in ditch enclosures differing in vegetation density. Pest. Manage. Sci. 60, 75–84.
39. Manna, S; Bhattacharyya, D.; Mandal, T.K. and Das, S. (2005): Repeated dose toxicity of deltamethrin in rats. Ind. J. Pharmacol., 37 (3): 160-164.
40. Matsumoto,M.Fukuyo,M.and Hara,Y. (1998): Effect of green tea catechins on plasma cholesterol level in cholesterol –fed rats. J.Nutr. SCi. Vitaminol., 44,337-342.
41. Mileson, B.E.; Chambers, J.E.; Chen, W.L.; Dettbran, W. and Ehrich, M. (1998): Common mechanism of toxicity: a case study of organophosphorus pesticides. Toxicol. Sci., 41:8-20.
42. Muthuviveganandavel, V.; Muthuraman, P. Muthu, S. Srikumar, K. (2008):a study on low dose cypermethrin induced histopathology, lipid peroxidation and marker enzyme changes in male rat. Pesticide Biochemistry and Physiology 91, 1, 12-16
43. Neishabouri, E.Z. Hassan, Z.M. Azizi, E. Ostad, S.N. (2004): Evaluation of immunotoxicity induced by diazinon in C57bl/6 mice, Toxicology 196:173–179.
44. Niedergethmann M., Hildenbrand R., Wostbrock B., Hartel M. (2002): High expression of vascular endothelial growth factor predicts early recurrence and poor prognosis after curative resection for ductal adenocarcenoma of the pancreas. Pancreas, 25: 122- 129.
45. Mechanick,J.I. (2006): Metabolic mechanisms of stress hyperglycemia. J.Parenter. enteral.Nutr.30:157-163.

46. Ohkawa, H.; Ohishi, N.; Yagi, K.(1979): Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (1979) 351–358.
47. Organization for Economic cooperation and development (OECD), chairman's Report of the meeting of the ad hoc working group of Experts on systemic short term and (delayed) neurotoxicity, 1992.
48. Poet, T.S.; Kousba, A.A.; Dennison, S.L; Timchalk, C. 2004: Physiologically based pharmacokinetic/ pharmacodynamic model for the organo- phosphorus pesticide diazinon, NeuroToxicology 25 , 1013–1030.
49. Poovala VS, Huang H, Salahudeen AK. 1999: Role of reactive oxygen metabolites in organophosphate induced renal tubular cytotoxicity. J Am Soc Nephrol 10:1746-52.
50. Price, R.J.; Scott, M.P.; Gidding, A.M.; Walters, D. G. and Lake, B.G. (2008): Effect of BHT, curcumin propyl gallate and thiabendazole on cytochrome p450 forms in cultured human hepatocytes. Xenobiotica 38(6): 574-86.
51. Rahimi, R., Abdollahi, M., 2007. A review on the mechanisms involved in hyperglycemia induced by organophosphorus pesticides. Pesticide Biochemistry and Physiology 88:115-21.
52. Ranjbar A, Pasalar P, Abdollahi M. (2002): Induction of oxidative stress and acetylcholinesterase inhibition in organophosphorous pesticide manufacturing workers. Hum Exp Toxicol 21: 179- 82.
53. Rao, G. and Rao, K. (1993): Inhibition of monoamine oxidase-A of rat brain by pyrethroids: an in vitro kinetic study. Mol. Cell. Biochem., 124:107-114.
54. Reitman, S. Frankel, S. (1957): Colorimetric estimation of AST & ALT activities, J. Lab. Clin. Med. 28:56–63.
55. Roberts, LJ; Morrow, JD (2000): Measurement of F2-isoprostanes as an index of oxidative stress in vivo. Free Radic Biol Med 28: 505-513.
56. Schermer, S. (1967): In Blood Morphology of Laboratory Animals, third ed.,
57. Schettler, G. Nussel, E. (1975): Method for triglycerides, Aeb. Med. Soz. Med. Prav. Med. 10 :25.
58. Shertzer HG, Bannenberg GL, Rundgren M, Moldeus P.(1991): Relationship of membrane fluidity, chemoprotection, and the intrinsic toxicityof butylated hydroxytoluene. Biochem Pharmacol. Sep 27;42(8):1587-93.
59. Skrzydlewska, E.; Ostrowska,J.;Farbiszewski,R.andMichalak,K.(2002).:Protective effectofgreenteaagainstlipidperoxidationintheratliverandbrain.Phytomidicine,9:232-238.
60. Soderlund,D.M.,Clark,J.M.;Sheets,L.P.;Mullin,L.S.;Piccirillo,V.J.;Sargent,D.;Stevens,J.T.; and Weiner,M.L. 2002 :Mechanismofpyrethroidneurotoxicity:implication for cumulative risk assessment. Toxicology 171:3-59.
61. Sogar SM, Yance D, Wong RK.(2008): Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer – Part 2. Current Oncology. 13(1): 1-9.
62. Storm, J.E., Karl, K.R., Doull, J. 2000: Occupational exposure limits for 30 organophosphate pesticides based on inhibition of red cell acetylcholinesterase, Toxicology 150, 1–29.
63. Tappel, A.L. (1973): Lipid peroxidation damage to cell components. Fed proc., 32: 1870-1874.
64. Tinoco R, Halperine D. 1998: Poverty production and health: inhibition of erythrocyte cholinesterase via occupational exposure to organophosphate insecticides in Chipas, Mexico. Arch Environ Health 53: 29- 35.
65. Trinder, P., 1959. determination of blood glucose using 4- aminophenazone. J. Clin. Path., 22,246.
66. Tuzmen,N.; Candan,N.; Kays,E. and demiryas,N. (2008): Biochemical effects of chlorpyrifos and deltamethrin on altered antioxidative defense mechanisms and lipid peroxidation in rat liver. Cell Biochem.Funct.26:119-124.
67. Verma RS. 2001: Chlorpyrifos-induced alterations in levels of thiobarbituric acid reactive substances and glutathione in rat brain. Indian J Exp Biol 39: 174- 77.
68. Vidyasagar J, Karunakar N, Reddy MS, Rajnarayana K,Surender T, Krishna DR. 2004 :Oxidative stress and antioxidant status in acute organophosphorus insecticide poisoning. Indian J Pharmacol ; 36: 76-79.

69. Yang, R.S.H., Hong,H.L. and Boorman, G.A. 1989. Toxicology of chemical mixtures: Experimental approaches, underlying concepts, and some results. Toxicology Letters 49, 183-197
70. Zhang, Q.; Tang, X.; Lu, Q.; Zhang, Z. and LE, A. (2006): Green tea extract and EGCG-3- gallate inhibits hypoxia and serum induced HIF-1alpha protein accumulation and VEGF expression in human cervical carcinoma and hepatoma cells. Mol. Cancer Ther. 5(5): 1227-1238.
71. Zhen, M.C.; Wang, Q.; Huang, X.h.; Cao, L.Q.; Chen, X.L.; and Zhang, L. (2007): Green tea polyphenols epigallocatechines-3-gallate unhibits oxidative damage and preventive effects on carbon tetrachloride- induced hepatic fibrosis. J. Nutr. Biochem. 18(12): 795-805.
72. Zhong, Z.; Froh, M.; Lehnert, M.; Schoonhoven, R.; Yang, L.; Lind, H.;Lemasters. J.J. Thorman, R.G. (2003): Polyphenols from camellia sinensis attenuate experimental cholestasis induced liver fibrosis in rats. Am. J. Physiol. Gastrointest. Liver Physiol. 285(5): 1004-10

2009/23/3

Fourier Transform Infrared Spectroscopic Characterization of Dergaon H5 Chondrite: Evidence of Aliphatic Organic Compound

[1,3] Bhaskar J. Saikia, [2] G. Parthasarathy and [3] N. C. Sarmah

[1] G B Pant Institute of Himalayan Environment and Development, Almora-263 643, India
[2] National Geophysical Research Institute, (CSIR), Hyderabad-500 007, India
[3] Department of Physics, Dibrugarh University, Dibrugarh-786 004, India
vaskar_r@rediffmail.com

Abstract: We report the spectroscopic investigations of the Dergaon H5 chondrite in the mid infrared region. Compositional characterization is presented with the help of X-ray fluorescence (XRF) spectroscopy. Fourier transform infrared (FTIR) spectroscopic study of Dergaon H5 chondrite exhibits prominent absorption band in 800-1100cm^{-1} region originating from valance vibration of SiO_4 tetrahedra. Particular interest is directed towards the presence of trace of organic compound in the spectral region around 2800-3000cm^{-1}, which is generally not observed in the meteorites of this type. The FTIR approach shows the organic material present in Dergaon is aliphatic functional groups CH_2 and CH_3. [Nature and Science. 2009;7(5):45-51]. (ISSN: 1545-0740).

Key words: Dergaon H5 chondrite; organic compounds; infrared spectroscopy

1. Introduction

Meteorites are some of the oldest remnants of the solar system available for laboratory studies. They are classified on the basis of their mineralogy, structure and chemical compositions (Oura et al 2002; Randa et al 2003; Ebihara et al 1996; Larimer 1971). The general mineral groups in meteorites are: silicates, metal, sulfides, oxides, phosphates and carbon compounds. The main classes of meteorites are: (i) stony meteorites constituting 92.8% of all meteorites, (ii) stony iron meteorites constituting nearly 1.5% and (iii) iron meteorites with abundance of 5.7%. Generally majority of primitive meteorites have small round mass of olivine or pyroxene called chondrules, they are commonly named chondrites. Chondritic meteorites are the oldest and most primitive rocks in the solar system. The primary divisions of chondrite classification are the carbonaceous, ordinary, and enstatite classes. The chondrites are the most numerous meteorite group, accounting for 87% of all meteorites observed to fall. The largest group of chondritic meteorites is known as the ordinary chondrites, account for 80% of all known meteorites (Philip et al 2000). Three subgroups of ordinary chondrites are identified, H group (high iron), L group (low iron), and LL group (low total iron, low metal). There is some evidence that carbon is frequently associated with Fe-Ni metal (McKinley et al 1981; Scott et al 1988; Brearley 1990; Cronin et al 1988), and carbon of unknown chemical form has been identified at the surface of metal and troilite grains in ordinary chondrites (Makjanic et al 1993). In unequilibrated ordinary chondrites (UOCs), the carbon content usually ranges from 0.2 to 0.6%, but reaches 1% in a few cases (Smail et al 2000). A numbers of authors suggested that the volatile organic compounds including aldehydes, amides, amines, mono and di-carboxylic acids, aliphatic and aromatic hydrocarbons, heterocyclic aromatics, hydroxy acids, ketones, phosphonic and sulfonic acids, fatty acids, purines, pyrimidines are present in meteorites (Cronin and Chang 1993; Palme 2000). These organic molecules are generally believed to be abiogenic (Cronin et al 1988), have been produced by chemical rather than biologic processing. Exogenous delivery of organics to the earth's surface could have been an important source of these molecules on the prebiotic earth.

The FTIR spectroscopy presents the advantage of being non-destructive technique; therefore, it is highly appreciated for analysing precious samples such as meteorites. This technique is the most powerful method to characterize the nature of carbonaceous matter present in the ordinary chondrite. In the present work, analysis of Dergaon H5 ordinary chondrite (Grossman and Zipfel 2001) using mid-infrared (transmittance) are presented in order to facilitate an understanding of the relative nature of the SiO_4 tetrahedra in 800-1100cm^{-1} (10μm) and 800-400cm^{-1} (20μm) region and characterized aliphatic functional CH_2 and CH_3 groups in the spectral region around 2800-3000 cm^{-1} (3.4μm). Composition is presented with the help of X-ray fluorescence (XRF) spectroscopy.

2. Experimental Method

The sample preparation was performed in ultra-clean conditions. To avoid surface contamination we remove the exterior. We took only pieces coming from its interior. The environmental contamination on the sample was checked by optical microscopy. Sample preparation tools were cleaned with conventional method and the samples were crushed following the standard procedure. The X-ray fluorescence (XRF) data on the Dergaon H5 chondrite were collected by using powdered homogenous sample in pellet form. The XRF study was performed on a Philip Magix XRF spectrometer PRO model PW 2440 in wavelength dispersive mode. Typical uncertainty involved is +/- 0.02 wt %. The thin sections of the sample have been characterized by electron microprobe analyzer. The experimental details were same as described elsewhere (Bhandari et al. 2005, Dhingra et al. 2004). We tried to minimize the grinding time to avoid the deformation of the crystal structure, the ion exchange and the water absorption from atmosphere. The iron part of the sample was separated with the help of a strong magnet. The powdered sample was homogenized in spectrophotometric grade KBr (1:20) in an agate mortar and was pressed 3mm pellets with a hand press. The infrared spectra was acquired using Perkin-Elmer system 2000 FTIR spectrophotometer with helium-neon laser as the source reference, at a resolution of 4 cm^{-1} .The spectra were taken in transmission mode in the region 400-4000 cm^{-1}. The room temperature was 29°C during the experiment.

3. Results and Discussions

The elemental composition of Dergaon H5 chondrite was determined by X-ray fluorescence (XRF) using the Dhajala (H3) meteorite as a standard. The classification, petrological and chemical characteristics of the Dergaon H5 chondrite has been reported by Shukla et al (2005). The compositional result of this study shows good agreements with the results of Shukla et al. The values of the major elements of Dergaon H5 chondrite is listed in Table 1(a). The only deviation that is readily evident is the distinctly to the K content compared to the previous workers. The olivine composition of the meteorite is presented in Table 1(b). The fosterite and fayalite compositions are Fo_{80} and $Fa_{19.33}$ respectively. Pyroxene composition of is approximately Enstatite, En_{80} Wo_3 $Fs_{16.9}$.

Table 1(a). Element composition of Dergaon Meteorite (Nd: Not determined.)

	Element (wt%)											
Sample	Si	Mg	Ca	Al	Fe	Ni	Mn	Cr	K	P	Na	Ti
This work	17.03	14.26	1.19	1.20	27.73	1.75	--	0.03	0.067	0.15	0.67	0.04
Shukla et al (2005)	Nd	13.6	1.08	1.09	27.30	1.82	2329 (ppm)	3705 (ppm)	352 (ppm)	Nd	7061 (ppm)	Nd

Table 1(b). Olivine composition and major mineral phase of the Dergaon Meteorite

	Oxides (wt%)							
Sample	SiO_2	Cr_2O_3	FeO	MnO	MgO	NiO	CaO	Total
Sample 1	39.030	0.047	18.400	0.455	41.372	--	0.294	99.568
Sample 2	38.900	0.047	18.734	0.475	41.512	--	--	99.668
Sample 3	39.297	--	18.999	0.465	41.774	0.063	0.261	100.859

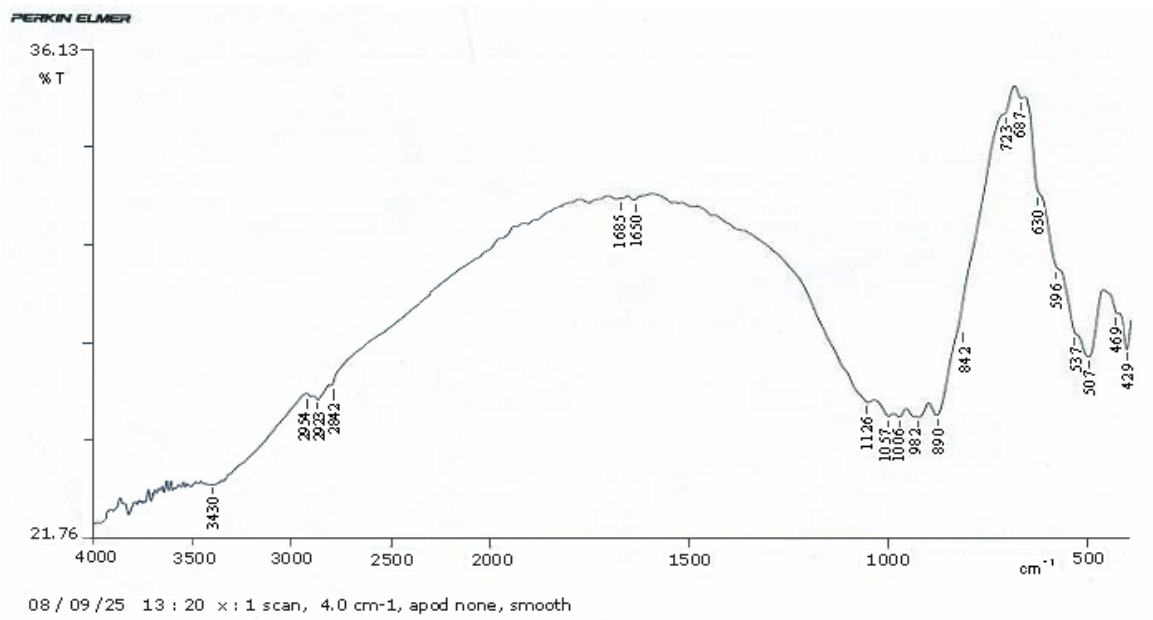


Figure 1. FTIR spectrum of the Dergaon meteorite in between 4000-400 cm^{-1} contains 10µm (800-1150cm^{-1}), 20µm (400-700cm^{-1}) and 3.4 µm (2800-3000 cm^{-1}) band. In 1150-400 cm^{-1} region, the spectrum exhibits four characteristics bands of olivine. The aliphatic stretching component is represented by the group of peaks in the 3000.2800 cm^{-1} region.

Table 2. Infrared peak positions in 10µm and 20µm region of Dergaon meteorite and their possible assignments are presented. Table indicates the four characteristic infrared peak positions of olivine group in the Dergaon meteorite (Wavenumber in cm^{-1}). The observed peak positions of Dergaon are compared with the olivine group i.e. fayalite, forsterite and enstatite according to the infrared data of Gadsden (1975).

Region	Fayalite (Fe_2SiO_4)	Forsterite (Mg_2SiO_4)	Enstatite (Mg_2SiO_3)	Dergaon	Assignments
	--	--	1128-04	1126	asymmetric stretching vibration, Si-O(TO2-T2O5)
	1060	1053	1070-56	1057	asymmetric stretching vibration, Si-O(TO2-T2O5)
	--	1000-990	1020-10	1006	asymmetric stretching vibration, Si-O(TO2-T2O5)
10µm	1012	965-55	965-58	982	Si-O asymmetric vibration, Si-O(TO3)
	976-82	982	950-47	--	--
	920-35	932	915-20	922	Si-O asymmetric vibration, Si-O(TO3)
	902-08	886-82	880-73	890	Si-O asymmetrical vibration, (T2O7- -TO4)
	852-77	--	--	--	Si-O asymmetrical vibration, (T2O7- -TO4)
	--	840	--	842	--
	828	--	--	--	--
	--	--	719-28	723	O-Si(Al)-O symmetrical Bending
20µm	--	--	693-95	687	O-Si(Al)-O symmetrical Bending
	--	620-02	--	630-596	--
	558-66	545	535	537	--
	510-02	512-01	505	507	Si-O and Mg-O vibration
	480-82	473-63	460	469	Si-O-Si bending vibration
	--	430-28	--	429	--

The infrared spectrum as shown in the Figure 1, reveals a number of absorption bands in the 10μm (800-1150cm^{-1}) and 20μm (400-700cm^{-1}) and it indicates the presence of silicates in the sample. The comparison of olivine group with (Gadsden 1975) Dergaon H5 ordinary chondrite is presented in the Table 2. The free SiO_4 ion has exhibit four fundamental vibrational modes: a symmetric stretch (v_1); a symmetric bend (v_2); an asymmetric stretch (v_3) and an asymmetric bend (v_4). The strong bands in the 10μm (800-1150cm^{-1}) region is identified as Si-O stretching and the bands present in the 20μm (400-700cm^{-1}) region is assigned as Si-O-Si bending vibrations. The bands found in the decreasing intensities in the Si-O stretching region and the Si-O-Si bending vibrations at 1057, 1006, 982, and 507 cm^{-1} is identical to the bands of fayalite (Fe_2SiO_4) ($Fa_{19.33}$), the bands found at 469, 507, and 1006 cm^{-1} is identical to the bands of forsterite (Mg_2SiO_4) and the bands found at 507, 537, 723, 982, 1006, 1057 and 1126 cm^{-1} are identical to the bands of enstatite (Mg_2SiO_3) (Gadsden,1975). The band found at 504 cm^{-1} can be interpreted as Si-O and Mg-O vibration modes in enstatite ($MgSiO_3$) with slight shifts in the matrix (Nakamoto 1978). The petrologic type-5 chondrites have two strong peaks at 982cm^{-1} and 537 cm^{-1} which may be related to Fe-O and Mg-O stretching modes (Nyquist et al 1971). In the infrared spectrum the Si-O asymmetric stretching vibration (TO2-T2O5) is observed in between the peaks 1006-1126 cm^{-1}. In between the peak position 982 cm^{-1} and 890 cm^{-1} we observed the Si-O asymmetric vibration (TO3) and (T2O7- -TO4) respectively. In the bending vibration region, the symmetrical bending vibration of O-Si (Al)-O is observed at the peak position 687 cm^{-1}. Another peak is observed at 469 cm^{-1} which is due to Si-O-Si bending vibration.

We observed some trace around 3600-3700 cm^{-1} region which may be due to O-H stretching of structural hydroxyl features of phyllosilicates. Generally OH groups reside at the octahedral surface of the layers and forms weak hydrogen bonds with the oxygen of the Si-O-Si bonds on the lower surface of the octahedral plane (Madejova et al 2001). The presence of very week band at 1650 cm^{-1} is attributed to H-O-H bending vibration. Another strong peak at 3430 cm^{-1} is arising due to the dissolved volatile species.

Table 3. Infrared band positions of 3.4 μm region and assignments of the Dergaon meteorite.

Sample	Wavenumber (cm^{-1})			Assignments
	Peak position	% Transmission	Intensity	
At room temperature	2954	33.0165	0.48127	CH_3 stretching vibration
	2923	31.8190	0.49313	CH_2 stretching vibration
	2842	33.2560	0.47813	CH_2 symmetric stretch
80°C after 24 hour	2956	12.4985	0.90314	CH_3 stretching vibration
	2921	12.3628	0.90788	CH_2 stretching vibration
	2862	12.7698	0.89382	CH_2 symmetric stretch
80°C after 48 hour	2926	24.9472	0.60298	CH_2 stretching vibration
	2858	25.8187	0.58807	CH_2 symmetric stretch

In 3.4 μm (2800 – 3000 cm^{-1}) region three major peaks are found corresponding to aliphatic hydrocarbon stretching features. The pair of peaks at 2922 and 2851 cm^{-1} corresponds to the asymmetrical and symmetrical stretching vibrations of CH_2 in an aliphatic hydrocarbon. The peaks at 2958 and 2865 cm^{-1} correspond to the asymmetrical and symmetrical stretching vibrations of CH_3 also in an aliphatic hydrocarbon (Salisbury et al 1992; Socrates 2001; Matrajt et al 2004). The sub-features of both symmetric and asymmetric C–H stretching vibrations of $–CH_2–$ and $–CH_3$ of aliphatic entitles with C–C single bonds. At room temperature, Dergaon meteorite shows weak absorptions of C-H stretching bends in between 2852–2972 cm^{-1}, indicating polyatomic entitles with C bonded to two or three H. The strongest ν_{CH} bend in between 2921–2926 cm^{-1} assigned to symmetrical stretch of C–H mode of $–CH_2–$ group. The bend in between 2842–2862 cm^{-1} is assigned to asymmetrical stretch of $–CH_2-$ group. Another bend is found at 2954–2956 cm^{-1} due to symmetric stretch of $–CH_3$ group (Table. 3). The variation of the peak intensity in

2800 – 3000 cm^{-1} region is observed by heating the pellet at a constant temperature 80°C with a time difference of 24 hours. The intensity of the peak shows a discrepancy with time. In the CH_3 stretching vibration around 2954-56 cm^{-1} significant changes observed after 48 hours (Fig.2 c). On further heating for 24 hours, the bands in this region are disappeared. There is no significance change is observed due to temperature treatment in the Si-O stretching and bending region. The weak absorption bands observed in the Dergaon meteorite are indicative to organic compounds present in the meteorite, the features observed in other H-chondrites by Lawless *et al*. (1972). The stretching features of aliphatic hydrocarbon are generally not observed in the meteorite of H types. The cause of weathering in the studied meteorite sample may be responsible for presence of aliphatic hydrocarbon stretching features. Further work on phase stability of CH stretching region and weathering nature of Dergaon meteorite are under progress.

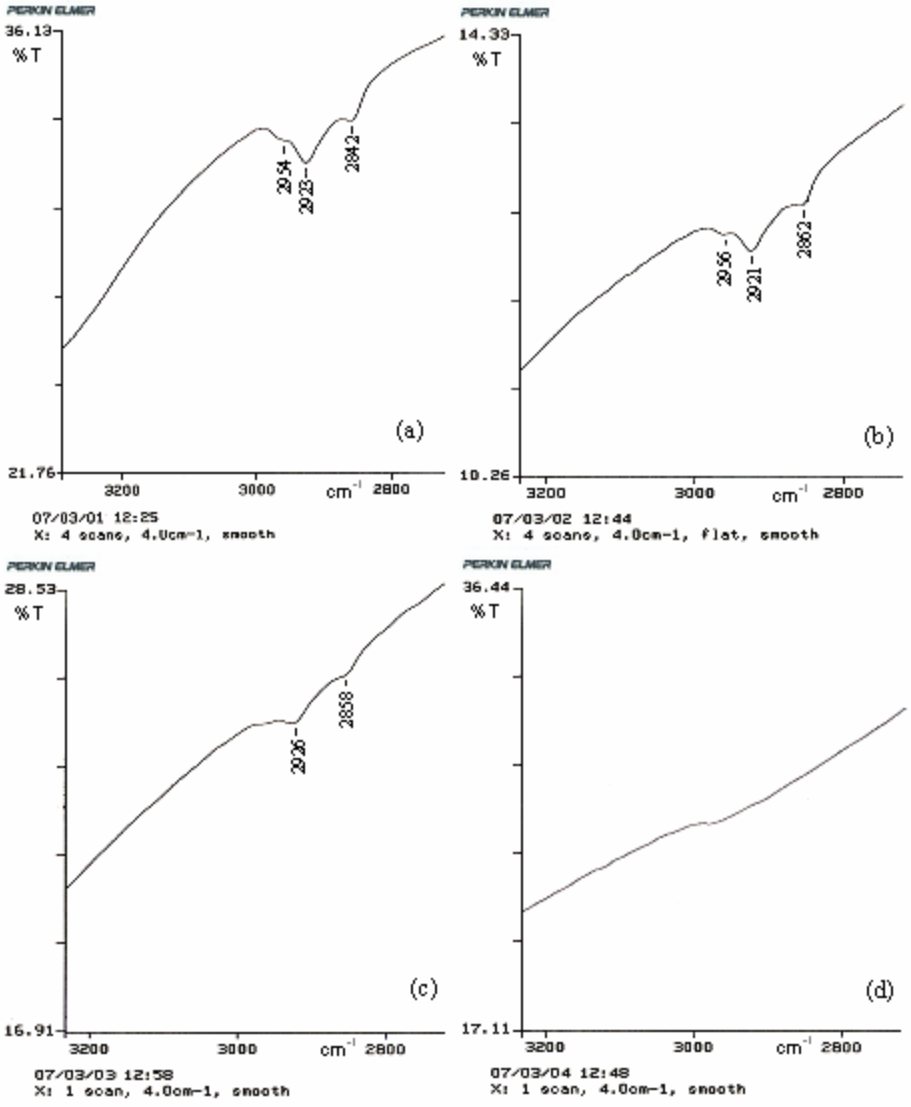


Figure 2. The infrared spectra in 2800-3000 cm^{-1}region exhibits aliphatic hydrocarbon in Dergaon meteorite. The spectra demonstrate the change of peak positions with temperature. (a) at room temperature (29ºC), (b) heating at constant temperature 80ºC for 24 hours, (c) heating at constant temperature 80ºC for 48 hours, (d) heating at constant temperature 80ºC for 72 hours. The spectra show the variation of peak intensities with time.

4. Conclusion

The data obtained during this work characterized the olivine group and aliphatic hydrocarbon (CH_2 and CH_3) in the Dergaon H5 chondrite. Prominent peaks at 1006, 982, 922, and 890$cm^{-1}$of infrared spectra correspond to the four characteristics band of olivine group present in the meteorite sample which is originated from the valance vibrations of SiO_4 tetrahedra, a basic component of the silicate lattice. The infrared band observed in the 2842-2962 cm^{-1} region is indicative to stretching features of aliphatic hydrocarbon. The weathering of the meteorite sample cannot be overlooked for presence of these features. If we ignore the terrestrial and atmospheric contaminations occurred due to sample collection and preservation, the presence of stretching features of aliphatic hydrocarbon is indicative to the organic compounds, which significant for astrobiology.

Acknowledgments

We thank Directors, National Geophysical Research Institute (NGRI), Indian Institute of Chemical Technology (IICT), Hyderabad for providing analytical facilities for characterization of the meteorite. One of us (GP) is grateful to ISRO, Department of Space, Government of India, for partial financial support under PLANEX program. We thank Prof. S.V.S. Murty, Coordinator, PLANEX, Department of Space, Government of India, for his valuable discussion. We also thank to Dr. P.K. Baruah, Gauhati University, Guwahati, for his assistance in the FTIR analysis.

Correspondence to: Bhaskar J. Saikia, e-mail:vaskar_r@rediffmail.com

References

1. Brearley A. J. Carbon-rich aggregates in type 3 ordinary chondrites: Characterization, origins, and thermal history, *Geochim. Cosmochim. Acta*, **54**, 831–850, 1990
2. Bhandari, N., Murthy, S.V.S., Shukla, P.N., Mahajan, R.R., Shukla, A.D., Suthar, K.M., Parthasarathy, G., and Paliwal, B.S., Bhawad LL6 chondrite : Chemistry, Petrology, noble gases, nuclear tracks, and cosmogenic radionuclides, *Meteoritics & Planetary Sciences*, **40**,1015-1021, 2005
3. Cronin, J. R., Chang, S., The Chemistry of Life's Origin (Eds.) J M Greenberg, CX Mendoza-Gomez, V Pirronello (Dortdrecht : Kluwer) 209-258,1993
4. Cronin, J.R., Pizzarello, S. and Cruikshank, D.P., Meteorites and the Early Solar System, University of Arizona Press, Tucson, 819-857, 1988
5. Dhingra, D., Bhandrai, N., Shukla, P.N., Murty, S.V.S., Mahajan, R.R., Ballabh, G.M., Lashkari, G., Shukla, A.D., Parthasarathy, G., Spectacular fall of the Kendrapara H5 chondrite, *Meteoritics & Planetary Sciences*, **39**, A121-A132, 2004
6. Ebihara, M., Shinotsuka, K. and Kong, P., Chemical composition of Martian meteorites. 61st Annual Meteoritical Meeting, Germany, 1996
7. Gadsden, J. A., Infrared spectra of Mineral and related Inorganic Compounds. USA: Butterworths. 1975
8. Grossman, J.N. and Zipfel, J. The meteorological bulletin, No.85, 2001, Sept. *Meteoritics & Planetary Sciences*, **36**, A293-A322, 2001
9. Lawless, J. G., Folsome, C. E., and Kvenvolden, K. A. , Organic matter in meteorites. *Scientific American* **226**, 38- 46, 1972
10. Larimer, J. W., Composition of the earth: chondritic or achondritic? *Geochim. Cosmochim. Acta*, **35**, 769–786, 1971
11. Madejova, J., Komadel, P. Baseline studies of the clay minerals society source clay: Infrared methods. *Clay and Clay Min*. **49**,410-432, 2001
12. Makjanic J., Vis R. D., Hovenier J. W., and Heymann, D. Carbon in the matrices of ordinary chondrites. *Meteoritics*, **28**, 63–70, 1993

13. Matrajt,G., Borg,J., Raynal, P. I., Djouadi, Z., d.Hendecourt, L., Flynn, G., and Deboffe, D.,FTIR and Raman analyses of the Tagish Lake meteorite: Relationship with the aliphatic hydrocarbons observed in the Diffuse Interstellar Medium, *Astronomy & Astrophysics*, **416**, 983.990, 2004
14. McKinley S., Scott E. R. D., Taylor G. J., and Keil K. A unique type 3 chondrite containing graphite-magnetite aggregates Allan Hills A77011. Proc. Lunar Planet. Sci. **12B**, 1039–1048, 1981
15. Nakamoto K. 1978, Infrared and Raman Spectra of Inorganic and Coordination Compounds, (3rd Ed.). New York. John Wiley & Sons
16. Nyquist, R. A. & Kagel, R. O., Infrared Spectra of Inorganic Compounds, 3800-45cm^{-1} Academic Press: New York, 1971
17. Philip, A.B., Alex W.R. B., Tim Jull, A. J., Ancient Meteorite Finds and the Earth's Surface Environment, *Quaternary Research***, 53**, 131–142, 2000
18. Oura, Y., Ebihara, M., Yoneda, S. and Nakamura, N., Chemical composition of the Kobe meteorite; neutron- induced prompt gamma ray analysis study. *Geochem. J*., **36**, 298–307, 2002
19. Plame,H., In From Dust to Terrestrial Planets (Eds.W.Benz R., et al.) Kluwer Academic Publishers, Boston, 237- 264, 2000
20. Randa, Z., Kucera, J. and Soukal, L., Elemental characterization of the new Czech meteorite 'Moravka' by neutron and photon activation analysis. *J. Radioanal. Nucl. Chem*., **257**, 275–283, 2003
21. Salisbury, J. W., Walter, L. S., Vergo, N., & D.Aria, D. M., Infrared (2.1.25 μm) spectra of minerals, The Johns Hopkins University, 1992
22. Scott E. R. D., Brearley A. J., Keil K., Grady M. M., Pillinger C. T., Clayton R. N., Mayeda T. K., Wieler R., and Signer P., Nature and origin of C-rich ordinary chondrites and chondritic clasts. Proc.Lunar Planet. Sci. Conf. 18th, 513–523, 1988
23. Shukla, P.N., Shukla, A. D., Rai, V. K.,Murty, S. V. S., Bhandari, N., Goswami, J. N., Mazumdar, A. C., Phukon,P., Duorah,K., Greenwood,R. E., and Franchi, I. A., The Dergaon (H5) chondrite: Fall, classification, petrological and chemical characteristics, cosmogenic effects, and noble gas records, *Meteoritics & Planetary Science*, **40**, 627–637, 2005
24. Smail, M., Claude, P., Ernst, Z. and Gerard S. Metal-associated carbon in primitive chondrites: Structure, isotopic composition, and origin, *Geochim Cosmochim Acta*, **64**, 1945–1964, 2000
25. Socrates, G., Infrared and Raman characteristic group frequencies, (3rd Ed.) Wiley and Sons, LTD., 2001

Response Of French Bean (*Phaseolus Vulgaris* L.) To Organic Manures And Inorganic Fertilizer On Growth & Yield Parameters Under Irrigated Condition

N. Indrakumar Singh[1] and J.S. Chauhan[2]

'1' Research Scholar, Department of Seed Science & Technology, H.N.B. Garhwal University, Srinagar Garhwal, (Uk)-246 174 (India). E. mail: indrasst08@gmail.com
'2' Assoc. Prof. & Head, Department of Seed Science & Technology, H.N.B. Garhwal University, Srinagar Garhwal, (Uk)-246 174 (India). E. mail: jai002004@yahoo.com

ABSTRACT: The investigation was conducted at field research centre of Department of Seed Science and Technology, H.N.B. Garhwal University, Srinagar (India) during Rabi season, 2007, on sandy loam soil, p^H having 5.3 to 5.5 to study the effect of organic sources of nutrients viz., vermicompost, FYM and along with inorganic fertilizers in French bean under irrigated condition with an objective to study growth and yield without degrading soil quality by using various nutrient compositions. In this investigation, vermicompost treatment (T2) recorded the highest in all observations except biomass of whole plant (above and ground biomass) which was recorded highest in N:P:K (T1) treatment this may be due to high composition of Nitrogen in inorganic fertilizers which supplement to the plant's vegetative phase. Thus it may be concluded that vermicompost was found useful than any other type of treatments under irrigated condition of Srinagar valley. [Nature and Science. 2009;7(5):52-54]. (ISSN: 1545-0740).

Key words: Vermicompost, FYM, N:P:K, Production, Seedling Growth.

Introduction

French bean (*Phaseolus vulgaris* L.) is one of the most important leguminous vegetables in India. It is a nutritious vegetable and can be grown in all types of soils ranging from light sandy loam to clay soils but it cannot withstand water-logging. The highest yield is obtained in soils with a P^H between 5.3 and 6.0. The use of chemical fertilizers boosted the agricultural products and the farming communities are using the same indiscriminately in such areas where irrigation facility exists with an eye on two to three crops in a year. This has drained the soil and resulted in the loss of soil productivity. So to obtain maximum return farmers need to apply high quantity of fertilizers and due to this culture the rate of use of fertilizers are increasing day by day, which means unlimited draining of soil. In spite of the importance for urgent step-up, very little attention has been paid so far to nutrient management in various soil and climatic conditions. The preparation and use of organic manures as a nutrient management may provide a hygiene and useful way of disposal and utilization of waste which would otherwise have created a healthy environment. Sankhyan et al. (2001) reported the increase in soil moisture due to mulching and significant increase in productivity of maize due to application of FYM. Kumaran (2001), reported the application of FYM+ fertilizer produced higher number of matured pods per plant, pod weight per plant, number of kernels per pod, test weight, pod yield and haulm yield of groundnut. But use of fertilizer alone recorded lower pod yield. Veerabhadraiah et al. (2006) showed improved soil properties due to application of either FYM or compost or vermicompost. Yadav and Vijayakumari (2003) found better yield in vermicompost treatment. Same observation was also reported by Rameshwar (2006). Guu et al. (1995) reported pod yield with fertilizer and manure application.

Keeping the views of the above aspects the present research work was, therefore, undertaken to find out the response of French bean to vermicompost, farmyard manure, N:P:K (Chemical fertilizer) and their different combination treatments under irrigated condition of Srinagar valley of Uttarakhand, India.

Materials and methods

The experiment was carried out at the field research centre of Department of Seed Science and Technology, H.N.B. Garhwal University, Srinagar, Uttarakhand (India) during Rabi season of the year 2007.

Experimental Site: Srinagar (Garhwal), 540 meter above of msl is situated between the latitude $36^0$12'24" to $30^0$13'24" North and longitude $78^0$41'22" to $78^0$49'42" east, it is a large valley about 6 Km long and 1.5 Km wide, spreading on both sides of the river Alaknanda. This valley exhibits subtropical extreme climate with dry summer and severe winter with occasional dense fog in early morning from December to mid March except

during rainy season. Rests of the months are usually dry with exceptions of occasional showers during winter or early spring. Minimum temperature ranges between 4^0C and 12^0C in the month of January. The soil of the site is sandy loam and clay soils with p^H value 5.3 to 5.5.
Inorganic fertilizers (N:P:K) which are widely used in agriculture and organic manures [farmyard manure (FYM) and vermicompost (Vc)] were used as materials. Method of cultural operation was adopted as per recommended practices. And observations were recorded mainly on growth (germination count, plant height, number of leaves, length of leaves, width of leaves, no. of pods per plant, root length, no. of nodulation) and yield (no. of seed per pod, yield per plot and biomass of shoot and root)

The experiment comprised of the following treatments:

Treatments	**Material(s) used**	**Quantity (Kg/bed)**
T1	N:P:K	0.10
T2	Vc	4.00
T3	FYM	6.00
T4	N:P:K + FYM	0.50+ 3.00
T5	Vc + FYM	2.00 + 3.00
T6	N:P:K + Vc + FYM	0.50 + 2.00 + 3.00
T7	Control	Nil
T8	N:P:K + Vc	0.50 + 2.00

Result

In all the observation aspects of growth the maximum value was recorded under vermicompost treatment (T2) (Table 1). But minimum value was found variably in different treatments and different observation aspects. The average production per plot was found highest (2.6 Kg/bed) and lowest (1.30 Kg/bed) in vermicompost treatment (T2) and mixed treatment of N:P:K+Vc+FYM (T6) respectively. The number of seed per pod was recorded maximum with same seed number (9.4/pod) in four treatments viz., vermicompost (T2), N:P:K+FYM (T4), Vc+FYM (T5), N:P:K+Vc+FYM (T6) treatments and minimum (8.4/pod) in control treatment (T7). The above and below ground dry biomass recorded the maximum average weight of above (55.74gm) and below (6.93gm) under T1 treatment (N:P:K).

Table 1. Mean effect of various levels of treatment on germination (Gm), height of plant (Ht), number of leaves per plant (NL), length of leaves (LL), width of leaves (WL), number of pods per plant (NPP), length of root (LR), number of Nodules (NN), above ground Dry biomass of plant (ADB), below ground dry biomass of plant (BDB), yield per plot (YP) and number of seeds per pod.

Treatments	Gm (%)	Ht (cm)	NL	LL (cm)	WL (cm)	NPP	LR (cm)	NN	ADB (g)	BDB (g)	YP (kg)	NSP
N:P:K (T_1)	80.0	22.73	20	17.93	10.84	20.6	29.24	57.2	55.74	6.93	1.990	9.0
Vc (T_2)	97.5	30.13	30	28.17	12.02	25.2	30.34	67.2	15.02	2.275	2.600	9.4
FYM (T_3)	85.0	23.67	24	25.60	9.50	21.6	27.44	64.6	20.52	2.095	2.000	8.6
N:P:K + FYM (T_4)	87.5	26.09	25	23.91	9.75	18.8	22.14	58.6	20.80	1.160	1.800	9.4
Vc + FYM (T_5)	87.5	23.02	24	24.63	9.95	20.4	21.28	56.0	18.99	0.549	2.100	9.4
N:P:K + Vc + FYM (T_6)	82.5	21.09	25	24.44	9.73	18.4	24.01	60.0	19.97	0.749	1.300	9.4
Control (T_7)	77.5	22.35	27	24.13	9.78	13.6	18.56	60.6	11.82	1.319	1.750	8.4
N:P:K + Vc (T_8)	75.0	21.31	24	21.46	9.06	15.0	17.30	54.0	14.79	0.757	1.900	9.2

Discussion

As per the findings of this investigation the result of the vermicompost treatment was found best than the farmyard manure, inorganic fertilizers and mixed treatments. Higher production per plot, germination percent, height of plant, number of leaves, length of leaves, number of pods per plant, length of root and number of nodules by farmyard manure (FYM) treatment than the N:P:K treatment support the findings of Rameshwar (2006) but contradicts the result of Pradhan and Mondal (1997). The high growth and yield recorded from FYM and FYM + Vc treatment in the experiment support the findings of Sankhyan et al. (2001) and Kumaran, (2001). The maximum overall growth and yield record from the vermicompost treatment and admixed with FYM were found consistent with the findings of Yadav and Vijayakumari (2003). The biomass of whole plant (above and ground biomass) was recorded highest in N:P:K (T1) treatment. The maximum biomass obtained may be due to high composition of Nitrogen in inorganic fertilizers which supplement to the plant's vegetative phase. The result was in accordance with the findings of Sharma et al. (2001). The experiment results revealed that the highest productivity by vermicompost, farmyard manure (FYM) and vermicompost + FYM treatments may be due to the improvement of physico-chemical properties of the soil and can be used as a resource for maximum crop productivity with more financial output in comparison to those chemical fertilisers. This observation was found consistent and accordance with the report of Veerabhadraiah et al. (2006).

From the above discussion, it may be concluded that Frenchbean was most responsive to vermicompost treatment (T2) on growth and yield in comparison to farmyard manure, chemical fertilizer and mixed treatments under irrigated condition of Srinagar valley. It also concluded that vermicompost is particularly good for farmers, consumers and ultimately for soil as it can be used as a resource for maximum crop productivity with more financial output in comparison to those chemical fertilisers. The highest productivity by vermicompost treatment may be due to the improvement of physico-chemical properties of the soil.

References

Guu J. W., Yang, T. C. and Fong, T. Y. Effects of organic manure on the growth and yield of common bean at fall season bulletin of Taichung. District Agricultural Improvement Station. 49: 41-48, 1995. (Cited from Horticultural Abstracts, CBA.1997. 67:4).

Kumaran, S. Response of groundnut to organic manure, fertilizer levels, split application of phosphorus and gypsum application under irrigated condition. Res. on Crops 2, 2001 (2) : 156-158.

Pradhan, B. K. & Mondal, S. S. Integrated nutrient management for sustaining productivity and fertility building of soil under rice based cropping system. Indian J. Agric. Sci., 1997, 67: 307-310.

Rameshwar, Dogra, V., Sharma, G. D. and Rana, S. Effect of FYM and sand application on Barley (*Hordeum vulgare*) under Barley – fallow cropping system in the cold desert region of Himachal Pradesh. Himachal J. of Agric. Res. 2006, 32 (2):22-24.

Sankhyan, N. K., Bharat Bhushan and Sharma, P. K. Effect of phosphorus, mulch and farm yard manure on soil moisture and productivity of maize in mid hills of Himachal Pradesh. Res. on Crops 2, 2001, (2) : 116-119.

Sharma, S., Daramwal, N. S., Sharma, C. R. and Upadhyay, R. G. Influence of various doses of nitrogen and phosphorus on protein content, yield and its attributes of mungbean [Vigna radiata (L.) Wilczek]. Res. on Crops 2 2001, (2) : 108-111.

Veerabhadraiah, T.N., Chamegowda and Badrinath, T.C. Effect of organic and inorganic sources of nutrients on avilabality and uptake of major and secondary nutrients by French bean in eastern dry zone of karnataka, India (Phaseolus vulgaris L.). The 18th World Congress of Soil Science (July 9-15, 2006), 115-49.

Veerabhadraiah,T.N., Chamegowda, and Badrinath, T. C. Cosequences of organic and inorganic sources of nutrients on physico-chemical properties of soil under French bean land use cover. The 18th World Congress of Soil Science (July 9-15, 2006), 163-20.

Yadav, H. and Vijayakumari, B. Influence of vermicompost with organic and inorganic manures on biometric and yield parameters of chilli [Capsicum annuum (L.) var. Plri]. Crop Res. 25 (2) : 236-243 (2003).

3/25/2009

Application of RAPD, isozyme and protein markers for characterization of rice (*Oryza sativa* L.) varieties

Anuradha Bhhartiya*, Kamal K. Pandae*, Harpal S. Mewari* and Pankaj Sah**
* Department of Biotechnology, Government Post Graduate College, Haldwani, Nainital, Uttarakhand State, (India).
** Department of Applied Sciences, Higher College of Technology, Muscat, (Sultanate of Oman)
anuradhagbp@gmail.com , kamalpandey@yahoo.com , pankaj@hct.edu.om

Abstract: Morphological characters are usually used to identify the crop varieties because they can easily be observed at phenotypic level and provide the unique identification of crop varieties (Smith and Smith, 1992). However, these traits are under the control of many genes and show instability due to interaction with environments, which restrict the reliable identification (Patterson and Weather up, 1984). On the other hand increased the number of genetically released varieties through the efforts of plant breeders have created phenotypic uniformity which has further imposed restrictions on utilizing the morphological traits as markers especially for crops where genetic base is narrow. Thus reliable identification of genotypes is difficult to achieve on this basis. The advancement is genetics, biochemistry and molecular biology have made available new tools based on protein and DNA complements of individuals and are common in use (Smith and Smith, 1992). Molecular characterization was undertaken for nineteen rice varieties using twelve RAPD markers. four isozyme markers and protein marker.A total of amplicons were obtained with the average of 5.6 bands per primer. Of these, 48 were found to polymorphic and level of polymorphism was 70.58%. The number of bands generated was more primer dependent than the genotype dependent and ranged from 1 to 11. The percentage of polymorph bands ranged from 50% (EO 1598, EO 1594 to 100% (EO 1600, EO 1596). Primer EO1593, EO1600. EO1602 generated primer specific bands with Pant Majhera Dhan 7, UPRI 17B, Govind and Pant Dhan 6, respectively. The dendrogram revealed Pant Majhera Dhan 7 to highly diverse from rest of the eighteen varieties. Jaccard's pair wise similarity coefficient values 19 genotypes were calculated and the range of genetic similarity was found to 0.65 to 0.92 with average of 0.75± 0.05. A dendrogram was generated by UPGMA cluster analysis based on Jaccard's similarity coefficients, which showed poor grouping of genotypes due to genetic similarity among them. [Nature and Science. 2009;7(5):55-63]. (ISSN: 1545-0740).

Keywords: RAPD, Protein Markers, Molecular Taxonomy.

INTRODUCTION:

Rice (*Oryza sativa L.*) is world's most widely cultivated food crop. In India it is grown on 44.6 m ha area with the production of 93 million tones of milled rice. Globally India ranks first in area under rice cultivation (44.6 mha) and second in production (93.9 mt) after China and contributes to 23.5% of world rice production. Rice plays a vital role in the national food security, as it constitutes staple food for two-third of the population supplying about 33% of food calories.

In India, it is more than foodstuff; it's an entire culture and is a basis of both biological and cultural diversity**.** The quality preferences of rice consumers have resulted in a wide diversity of varieties specific to different localities. Although the exact diversity cannot be gauged, it is estimated to be around 140,000 different genotypes. India alone has 86, 330 accessions, of which 42,004 are in the national gene bank.

About 760 high yielding varieties have been released in India for various ecosystems. Thus varietal identification has attained a critical importance in India in view of increasing multiplicity of varieties and imminent implementation of Plant Variety Protection and Farmer's Rights Act, 2001. A breeder's variety must fulfill the criteria of Distinctness, Uniformity, and Stability (DUS) to be given protection under this Act. It is thus imperative to characterize all popular varieties and to prepare and maintain their comprehensive database.

However, to protect the rights of breeders/breeding institutions against misappropriations and plagiarization, it is desired that they are properly characterized on the basis of morphological and molecular characteristics. Gel electrophoresis of protein, RAPD markers and isozymes are a powerful tool to distinguish genotypes of plant species. Proteins being the last gene products reflect the genomic composition of varieties and hence are good candidates for varietal distinctness. These can provide useful supplementary information, which combined with morphological descriptors provide identification keys.

The present study was conducted to understand the pattern of genetic variability in nineteen rice varieties based on RAPD markers. For RAPD analysis, genomic DNA was isolated from fresh leaves (Murray *et al.,* 1980). One gm of fresh leaves of each of the nineteen varieties were crushed with a pre-chilled pestle and mortar to a fine powder for DNA extraction buffer (5 M NaCl, 0.5 M EDTA, pH=8, 1 M Tris base,PH=8, 10% CTAB). The homogenate was centrifuged to remove cell debris. The supernatant was treated with RNAse and DNA was precipitated with chilled ethanol. The quantity and quality was assayed by running DNA on a 0.8% agarose gel alongside a known quantity of lambda uncut DNA. Amplification reaction was carried out in 25µl reaction volume containing 75 ng of template DNA, 100ng/ µl of primer; 200 µM of dNTPs, 6U/ µl Taq (Bangalore Genei Pvt Ltd, Bangalore, India and 10X Taq buffer with $MgCl_2$). Twelve decamer primers were used for RAPD amplifications with minor modification. Amplification cycle consisted of denaturation for 1 min at 94^0C, 2 min of annealing at 36^0C followed by a 2 min at 72^0C, and primer elongation for 1 min at 72^0C. The PCR products were separated/resolved by electrophoresis on 1.5 % agarose gel from Genei, 1 X TBE buffer and ethidium bromide stained gel was photographed with a digital gel documentation system. Reproducibility of RAPD assay was tested by performing duplicate reaction at different times using identical genotypes and primer combinations under strict control of experimental condition and only the reproducible bands were scored. The RAPD bands were scored as present (1) or absent (0) for each genotype primer combination for all the nineteen rice genotypes, considering each amplified band as a unique locus. Band sharing data were used to calculate genetic similarities based Jaccard's coefficient (Jaccard, 1908) and UPGMA (Unweighted pair group method using arithmetic averages). Aloagrithim was employed to determine the genetic relationship of nineteen varieties (Sneath, 1973). All the analysis was performed using NTSYS-pc 2.02 software (Rholf, 2002).

For isozyme study, 7 days old etiolated seedlings were ground with 50mM tris Hcl buffer (PH=7.6) containing2mercaptoethanol and EDTA in ratio 1:2 (w/v). For extraction of Peroxidase, the buffer without 2 mercaptoethanol was used. Homogenate centrifuged at 15,000 x g and supernatants obtained were used for studying isozyme pattern respectively.

Isozymes were separated on 7% polyacrylamide gel using an anionic system [davis1964] and stained as described by Vallejos (10) for esterase[EST], peroxidases[POX], malate dehydrogenases[MDH] and alcohol dehydrogenases[ADH]. The bands were scored for construction of dendrogram using Jaccard's index. Data were entered as presence/ absence of bands ignoring the intensity for isozyme analysis.

Observations and Results:

Table A. The number of RAPD loci detected by using 12 RAPD primers on agarose gel (1.5%)

Code No.	Primer	Sequence	Total number of RAPD loci	Polymorphic loci	
				Number	%
1	EO1591	5'CACAGGCGG3'	10	8	80
2	EO1592	5'GTGTGCCCCA3'	7	5	71.4
3	EO1593	5'GGGAATTCGG3'	7	5	71.4
4	EO1594	5'CTGACCAGCC3'	4	2	50
5	EO1595	5'GGAAGTCGCC3'	7	5	71.4
6	EO1596	5'GGGAGACATC3'	4	4	100
7	EO1597	5'ACCGCGAAGG3'	1	0	0
8	EO1598	5'CCCGCCGTTG3'	6	3	50
9	EO1599	5'CCGCCCAAAC3'	3	0	0
10	EO1600	5'GTGCAACGTG3'	8	8	100
11	EO1601	5'GTTTCGCTCC3'	5	3	60
12	EO1602	5'TGCTGCAGGT3'	6	5	83.3

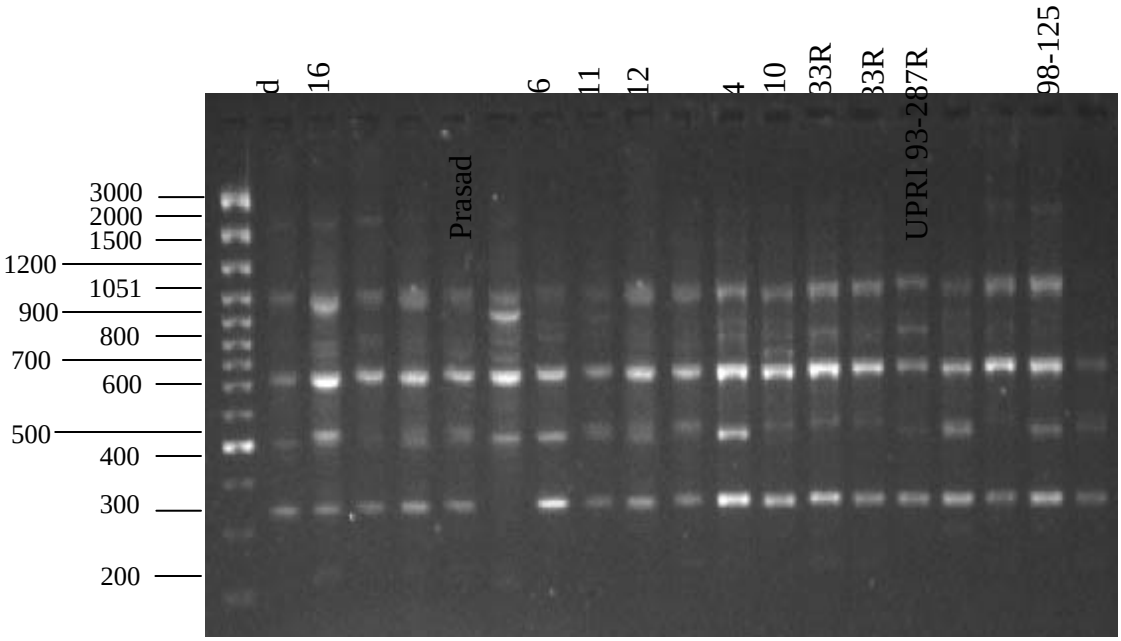


Plate.1. Banding profile of amplified DNA sequences from a RAPD reaction using Primer EO1591

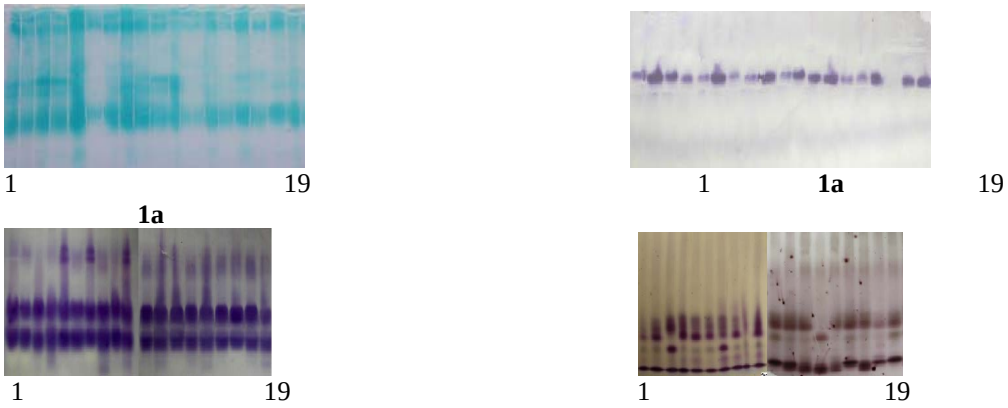


Plate.2. Isozyme pattern of seven days old seedlings of the 19 rice varieties.
1a. POX(Peroxidase),1b. ADH(alcohol dehydrogenase), 1c. MDH(Malate dehydrogenase), 1d.EST(Esterase)

Varieties: *Govind,. Manhar, Prasad, Pant Dhan* 4,*Pant Dhan* 6,*Pant Majhera Dhan* 7,*Pant Dhan* 10,*Pant Dhan* 11,*Pant Dhan* 12,*Pant Dhan* 16,*Pant Sankar Dhan* 1,*Pant Sankar Dhan* 3,*Saryu* 52,NDR 359,UPRI 92-133R,UPRI 95-17B,UPRI 93-287R,UPRI 99-1,UPR 2870-98-125

S.No.	Variety	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1.	Govind	1.00																		
2.	Pant Dhan 16	0.80	1.00																	
3.	Pant Sankar Dhan 1	0.84	0.77	1.00																
4.	Pant Sankar Dhan 3	0.75	0.81	0.82	1.00															
5.	Prasad	0.7	0.73	0.77	0.74	1.00														
6.	Pant Majhera Dhan 7	0.71	0.68	0.72	0.69	0.68	1.00													
7.	Pant Dhan 6	0.76	0.87	0.77	0.81	0.76	0.77	1.00												
8.	Pant Dhan 11	0.71	0.74	0.69	0.75	0.67	0.75	0.77	1.00											
9.	Pant Dhan 12	0.73	0.66	0.74	0.74	0.66	0.70	0.76	0.77	1.00										
10.	Maxhar	0.79	0.75	0.83	0.80	0.75	0.80	0.86	0.83	0.82	1.00									
11.	Pant Dhan 4	0.80	0.80	0.75	0.78	0.76	0.75	0.84	0.74	0.73	0.82	1.00								
12.	Pant Dhan 10	0.80	0.79	0.87	0.84	0.75	0.74	0.86	0.76	0.78	0.92	0.86	1.00							
13.	UPRI 92-133R	0.69	0.65	0.76	0.73	0.78	0.70	0.75	0.79	0.78	0.84	0.72	0.84	1.00						
14.	UPRI 95-17B	0.67	0.67	0.69	0.72	0.67	0.72	0.77	0.75	0.70	0.80	0.74	0.80	0.79	1.00					
15.	UPRI 93 287R	0.65	0.74	0.72	0.79	0.74	0.69	0.78	0.75	0.74	0.76	0.75	0.77	0.76	0.75	1.00				
16.	NDR 359	0.74	0.76	0.71	0.78	0.70	0.78	0.80	0.81	0.80	0.82	0.77	0.76	0.72	0.74	0.85	1.00			
17.	UPRI 99-1	0.75	0.75	0.76	0.76	0.82	0.80	0.82	0.76	0.71	0.81	0.75	0.78	0.80	0.76	0.76	0.82	1.00		
18.	UPR 2870-98-125	0.67	074	0.66	072	0.67	0.69	077	0.78	0.66	0.76	0.74	0.70	0.69	0.68	0.79	0.78	0.76	1.00	
19.	Saryu 52	0.69	0.68	0.70	0.73	0.65	0.67	0.72	0.76	0.71	0.81	0.69	0.75	0.70	0.69	0.77	0.76	0.71	0.80	1.00

Table 1. Pair wise similarity matrix based on Jaccard's coefficient or rice varieties by RAPD analysis

S.No.	Variety	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1.	Govind	1.00																		
2.	Pant Dhan 16	0.83	1.00																	
3.	PSD 1	0.83	0.83	1.00																
4.	PSD 3	0.84	0.84	0.84	1.00															
5.	Prasad	0.83	0.83	0.83	0.84	1.00														
6.	PMD 7	0.81	0.66	0.66	0.69	0.66	1.00													
7.	Pant Dhan 6	0.81	0.66	0.66	0.69	0.66	1.00	1.00												
8.	Pant Dhan 11	0.66	0.66	0.53	0.69	0.53	0.80	0.80	1.00											
9.	Pant Dhan 12	0.81	0.66	0.66	0.69	0.66	1.00	1.00	0.80	1.00										
10.	Manhar	0.84	0.86	0.84	1.00	0.84	0.69	0.69	0.69	0.69	1.00									
11.	Pant Dhan 4	0.61	0.61	0.75	0.76	0.61	0.72	0.72	0.72	0.72	0.76	1.00								
12.	Pant Dhan 10	0.61	0.61	0.75	0.64	0.61	0.72	0.72	0.58	0.72	0.64	0.81	1.00							
13.	UPRI 92-133R	0.78	0.78	0.78	0.92	0.78	0.64	0.64	0.64	0.64	0.92	0.71	0.71	1.00						
14.	UPRI 95-17B	0.61	0.61	0.50	0.64	0.50	0.72	0.72	0.90	0.72	0.64	0.66	0.66	0.71	1.00					
15.	UPR 93- 287R	0.84	0.84	0.84	0.85	0.84	0.69	0.69	0.57	0.69	0.85	0.64	0.76	0.92	0.64	1.00				
16.	NDR 359	0.91	0.91	0.91	0.92	0.91	0.75	0.75	0.61	0.75	0.92	0.69	0.69	0.85	0.57	0.92	1.00			
17.	UPRI 99-1	0.76	0.76	0.76	0.78	0.76	0.61	0.61	0.50	0.61	0.78	0.57	0.69	0.85	0.57	0.92	0.84	1.00		
18.	UPR 2870-98-125	0.91	0.91	0.91	0.92	0.91	0.75	0.75	0.61	0.75	0.92	0.69	0.69	0.85	0.57	0.92	1.00	0.84	1.00	
19.	Saryu 52	0.69	0.69	0.69	0.84	0.69	0.81	0.81	0.81	0.81	0.84	0.90	0.75	0.78	0.75	0.71	0.76	0.64	0.76	1.00

Table 2: Pair wise similarity matrix based on Jaccard's coefficient of rice varieties by isozyme analysis

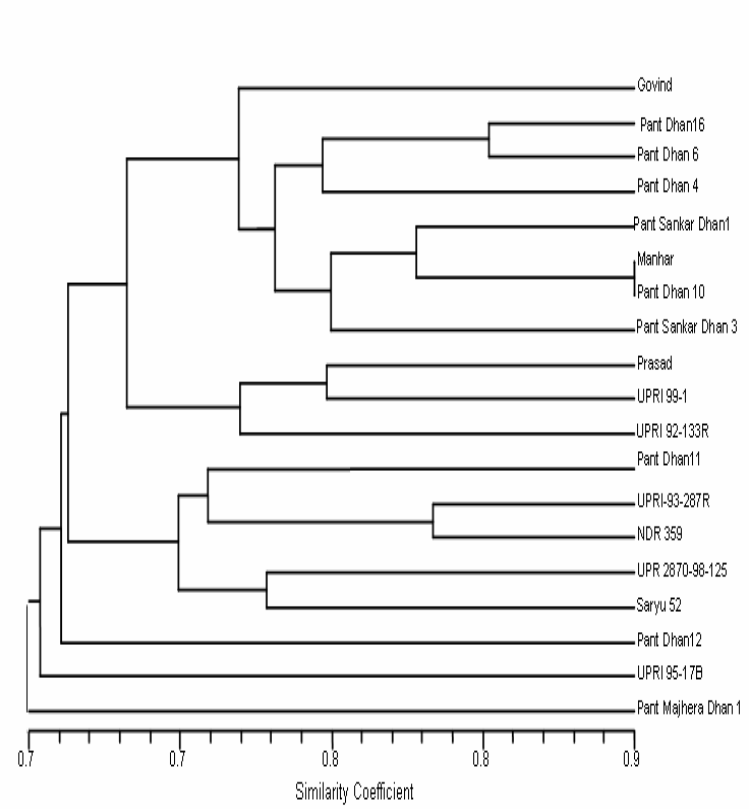


Fig. 1. Dendrogram of rice varieties based on RAPD analysis.

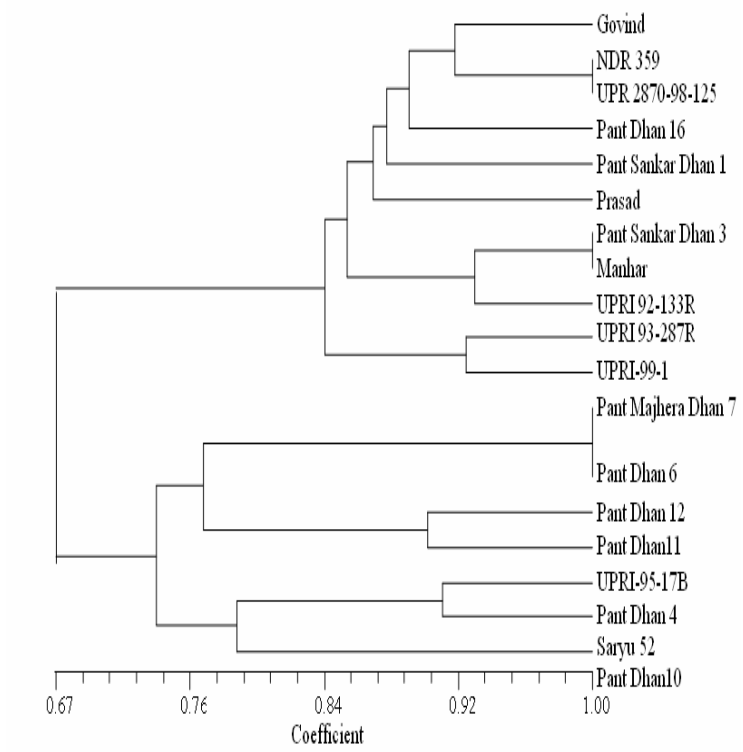


Fig. 2: Dendrogram of rice varieties based on isozymes (peroxidase, esterase, malate dehydrogenase and alcohol dehydrogenase)

Discussion:

PCR amplification of DNA extracted from nineteen varieties was done following the same protocol for all 12 RAPD primers. Before amplification with primers, optimization was carried out and it was observed that 75ng/µL DNA sample and 100 ng/µL concentration of primer was best. A typical example with primer EO1591 is shown in **Plate1**. A total of 68 amplicons were obtained with this primer with an average of 5.6 bands per primer. The number of bands generated was more primer dependent than genotype and ranged from 1 (EO1597) to 10 (EO1591). Out of these 68 bands, 48 bands were polymorphic and the level of polymorphism was 70.59%. **(TableA).** Two primers (EO1596, EO1600) out of twelve showed 100% polymorphism, while two primers namely EO1597, EO1599 did not show any polymorphism. Primer EO 1597 was found to be monomorphic as it gives single band. Various authors have also reported the variation in total bands produced and level of polymorphism generated**.** All the 19 genotypes have been discriminated in the present RAPD analysis involving twelve decamer primers. Though it is difficult to prepare a key for identification of all the varieties on the basis of polymorphism involving a small set of primers, a few primers have generated a very specific bands for only a limited number of genotypes. Primer EO1591, EO1593, EO1600, EO1602 generated primer specific bands with *Pant Majhera Dhan 7*, UPRI-95-17B, *Govind* and *Pant Dhan 6* respectively. Jaccard's pair wise similarity coefficient values for nineteen varieties were also calculated. The range of genetic similarity was found to be 0.65 to 0.92 **(Table1)**. The average similarity index based on Jaccard's coefficient was 0.75 $\pm$ 0.05. Overall view suggested that the grouping of the varieties is very poor as the similarity among the varieties. The dendrogram **(Fig.1)** revealed *Pant Majhera Dhan* 7 to be highly diverse from rest of the nineteen varieties since it showed only 73% similarity with rest of the group as it is selection from local germplasm of Pithoragarh district. The dendrogram further delineated the above varieties into two groups, by clearly demarcating UPRI 95-17B as highly distant from rest of the varieties by showing only 73.4% similarity with them.

All the varieties except *Pant Dhan* 12, *Pant Majhera Dhan* 7, and UPRI 95-17B divided into two major clusters 1 and 2 related at only 74.2% similarity level. Cluster 1 containing six genotypes and it were further divided into two sub clusters A and B being differentiated at 78% similarity between them. Cluster

A comprised of two genotypes *Saryu* 52 and UPRI 2870 95-125, sub cluster B was comprised of *Pant Dhan 11*, UPRI 95-287R, and NDR-359. UPRI 93-287R and NDR-359 with 86% similarity with each other. Cluster 2 had eleven varieties *Govind, Pant Dhan* 16, *Pant Dhan* 6, *Pant Dhan* 4 *Pant Sankar Dhan* 1, *Manhar*, *Pant Dhan* 10, *Pant Sankar Dhan* 3, *Prasad*, UPRI 99-1 and UPRI 99-133R and it was further divided into two sub clusters C and D differentiated with 76.3% similarity between them. Cluster C had three varieties UPRI 95-133R, UPRI 99-1 and *Prasad*. UPRI 99-1 showed relatedness with *Prasad* at 83% similarity level. Sub cluster D was again subdivided into two mini clusters with 81% similarity. One mini cluster had *Pant Sankar Dhan* 3, *Pant Dhan* 10 and *Manhar* with 83% similarity. *Manhar* showed close relation with *Pant Dhan* 10 (92% similarity) and seems to be identical as depicted in dendrogram , however it is impossible to prove that two varieties are genetically identical without sequencing their genomes. Varieties that are found identical or similar based on molecular analysis data may differ in just one important character. The other mini cluster had three genotypes *Pant Dhan* 4, *Pant Dhan* 6 and *Pant Dhan* 16. *Pant Dhan* 6 showed close relationship with *Pant Dhan* 16 with 87.7% similarity. The present study revealed existence of sufficient genetic variation at DNA level in nineteen varieties. This information will help breeder to characterize the varieties on the basis of polymorphism in addition to morphological marker, to select the diverse varieties based on variation at the DNA level to be used in crossing programme for realization heterosis identifying donor parents for useful agronomic attributes and markers linked to different polygenic traits.

Data scored from nineteen rice varieties with four isozymes (esterase, peroxidase, malate dehydrogenase and alcohol dehydrogenase) were used to generate Jaccard's pairwise similarity matrix. According to this, similarity coefficient ranged from 0.5 to 1.00. **(Table.2)** represents the similarity index among the varieties and **Fig.2** represents the dendrogram drawn on the basis of Jaccard's coefficient.

According to cluster analysis, there were two major cluster groups. The closely related varieties were grouped in one major group. Between two major cluster groups there is not too much distance observed. This showed that these varieties are closely related at biochemical level. This was entirely unexpected. The dendrogram was divided into two major clusters namely, cluster 1 and cluster 2, related at 67% similarity level. Cluster 1 was divided into two sub clusters, A and B differentiated at 73.5% similarity level. Sub cluster A contained three varieties namely, *Pant Dhan* 4, *Saryu* 52 and *Pant Dhan* 12. the variety *Pant Dhan* 10 was related to *Saryu* 52 and *Pant Dhan* 4 at 78.5% similarity level. There were five varieties in sub cluster B *viz.*, UPRI 95-17B, *Pant Dhan* 11, *Pant Dhan* 12, *Pant Dhan* 6 and *Pant Majhera Dhan* 7. Out of these five varieties, *Pant Majhera Dhan* 7, *Pant Dhan* 6 and *Pant Dhan* 12 showed 100% similarity with each other. *Pant Dhan* 11 and UPRI 95-17B showed 89.5% similarity with each other.

Cluster 2 was divided into two sub clusters C and D. Sub cluster C comprised of five varieties *viz*., UPRI 92-133R, UPRI 99-1, UPRI 93-287R, *Pant Sankar Dhan* 3 and *Manhar*.

Sub cluster C was comprised of UPRI 99-1 and UPRI 93-287R which were closely related (92%).

Sub cluster D is divided into two min clusters d` and d``. Mini cluster d` had six varieties *viz.*, *Govind*, NDR 359, UPR 2870-98-125, *Pant Dhan* 16, *Pant Sankar Dhan* 1 and *Prasad*. *Prasad* showed 85.5% similarity with *Govind*, NDR 359, UPR 2870-98-125, *Pant Dhan* 16 and *Pant Sankar Dhan* 1. Out of these six varieties of this sub cluster NDR 359 and UPR 2870-98-125 showed 100%
relatedness with each other and these varieties were related with *Govind* at 91.5% similarity level. *Pant Sankar Dhan* 1 was related with *Govind*, NDR 359, UPR 2870-98-125 and *Pant Dhan* 16 at 87% similarity level. Mini cluster d" consisted of *Pant Sankar Dhan* 3 and *Manhar* which showed 100% similarity with each other. The homology of genotypes (% similarity) for protein bands varied from 50 to 100% among different varieties. Results on homology between the varieties are presented in **Table 4.4.3.** Varieties NDR 359 and *Pant Dhan 4*, UPRI95-287R with *Pant Dhan 10* and UPRI 95-17B with *Pant Dhan10* and *Pant Dhan 6* with *Govind* showed complete homology of 100%.

Fig.4.4.2. depicts the relationship among nineteen rice varieties under study in the form of dendrogram. On the basis of cluster analysis, the varieties were divided into two major groups (Cluster 1 and 2). Within one major group there were closely related varieties. However, there was not too much distance between two major groups. This showed that these varieties were closely related at biochemical level. This was not entirely unexpected because *O. sativa* is an autogamous crop. Cluster 1 and 2 were related at 69% similarity level.

In cluster 1, UPRI 92-133R is related to other genotypes (*Pant Dhan 4*, NDR 359, UPRI 95-17B, UPR 2870-98-125, *Pant Dhan* 10, UPRI 93-287R, UPRI 99-1 and *Saryu* 52) at 75.5% similarity level. Saryu 52 has 80% similarity to rest genotypes in cluster 1. The dendrogram depicted 100% similarity

among *Pant Dhan* 10, UPRI 95-17B and UPRI 93-287R at biochemical level. The similarity observed among *Pant Dhan* 4, NDR 359 and UPR 2870-98-125 was 100%.

In cluster 2, Prasad was 74% similar to *Pant Majhera Dhan* 7, *Manhar*, *Pant Dhan* 12, and *Pant Sankar Dhan* 1. *Pant Sankar Dhan* 3, *Pant Dhan* 11, *Pant Dhan* 16, *Pant Dhan* 6 and Govind.

Cluster 2 was further divided into two sub clusters, A and B, differentiated at 79.5% similarity level. Sub cluster A consisted of six varieties, *Pant Majhera Dhan* 7 is related to *Manhar*, *Pant Dhan* 12, *Pant Sankar Dhan* 1, *Pant Sankar Dhan* 3 *and Pant Dhan* 16 at 81.5% similarity level. *Pant Sankar Dhan* 1 and *Pant Sankar Dhan* 3 were 92% similar with each other. In sub cluster B, *Govind* and *Pant Dhan* 6 exhibited 100% similarity with each other.

ACKNOWLEDGEMENT:

The authors are grateful to Head, Genetics and Plant Breeding Department, the Director of Experiment Station and Dean, College of Agriculture, G.B. Pant Agricultural University for providing the necessary facilities for conducting this investigation.

Authors for correspondence:

anuradhagbp@gmail.com , kamalpandey@yahoo.com , pankaj@hct.edu.om

References:

Davis J. (1964). Disc 3. Electrophoresis-II. Methods and application of human serum protein. *Ann.N.Y.Acad.sci*.121:404-427.

Jaccard P. (1908). Nouvelles resecherches sur la distribution florale. *Bull. Soc. Vaud Nat*., **44:** 223-270.

Murray, M. and Thompson, W. (1980). The isolation of high molecular weight plant DNA. *Nucleic acid Res*. **8**: 4321-4325

Rholf F.J. (2002). NTSYSpc: Numerical Taxonomy. System, ver 2.1. Exeter Publishing, Ltd.: Setauket, NY.

Sneath P.H.A. and Sokal R.R. (1973). Numerical Taxonomy. W.H. Truman and Co., San Fransisco, USA..

Williams J.G.K., Kuubelik A.R., Livak K.J. Rafalski A. and Tinglery S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acid Res*., **18**: 6231-6235.

Vallejos,E.(1983). Enzyme activity staining. In:S.D. tanksley and T.J. Orton(eds.).isozyme in Genetics and plant Breeding.Part A,pp.469-516.*Elseevier Science Publishers,B.V.Amsterdum*.

3/27/2009

Study on Seed Germination and Growth Behavior of Brinjal in Admiration to Effect of NPK and Organic Manure

Anoop Badoni* and J. S. Chauhan**
Seed Testing Laboratory
Department of Seed Science and Technology
H. N. B. Garhwal University, Srinagar Garhwal, 246 174, Uttarakhand
*Research Scholar, Young Scientist (UCOST)
**Assoc. Prof. and Head of Department
*For correspondence: annabadoni@yahoo.co.in

ABSTRACT
An experiment was conducted to study the seed germination and growth behavior of brinjal (*Solanum melongena* L.) with inorganic fertilizers (NPK) and organic manure (Cow dung) under environmental conditions. Seeds of *Solanum melongena* L. cv. BR 112, were sown in poly bags (1 seed/poly) at the depth of 2.5 cm. with different treatments i.e. S1 (Control- Only Soil), S2 (Soil + NPK), S3 (Soil + Cow dung). 50 replicates of each treatment were used for the study. Total numbers of germinated plants were counted from each poly bag of all treatments, at the interval period of 5 days after sowing, and reported as emergence count/poly bag. For growth study plant height, number of leaves, length and width of leaves and root length were measured from all the treatments. Result revealed that cow dung showed maximum germination% i.e. 49 plantlets from 50 seeds then control and NPK i.e. 29 plantlets and 35 plantlets respectively. Growth of plantlets also showed maximum plant height (59.2 cm.), number of leaves (5.8), length of leaves (7.82 cm.), width of leaves (5.73 cm.) and root length (19.63 cm.) in S3 treatment then control (34.6 cm. plant height, 3.7 number of leaves, 3.53 length of leaves, 2.72 width of leaves, 7.05 root length) and NPK (46.4 cm. plant height, 3.4 number of leaves, 4.15 length of leaves, 3.18 width of leaves, 17.76 root length). [Nature and Science. 2009;7(5):64-66]. (ISSN: 1545-0740).

Key words: *Solanum melongena* L., cow dung and NPK

INTRODUCTION:

Brinjal is used in all over the world as an edible vegetable crop. Brinjal or egg-plant (*Solanum melongena* L) is one of the most commonly grown vegetable crops of solanaceae family in India. India, China, Turkey, Japan, Philippines are the major brinjal production countries. India contributes 6,44,3062 MT to the global production of brinjal and ranks 2nd to China (Thamburaj and Singh, 2003). In Uttarakhand hilly regions it is grown only in summer. As we know the population of India increases day by day and by this region the scarcity of food also increases. To fulfill all human needs or to meet the demand of today's peoples, farmers generally used inorganic fertilizers to increases the quality and quantity of the crop. Although using these fertilizers farmers increases the yield of crops but this create an adverse effect on crops, consumer health and as well as on environment (Biswas and Mukharjee, 1994).

Traditionally in our villages and rural areas organic manure are used such as dung of domestic animals. Cow dung shows no or less adverse effect on crops and also on human health. The main scope of this investigation is that, the use of organic manure is better for quality and yield of the crops. As early as 5000 B.C. the *Vedas* and *Upanishads* as well as other Indian documents mention soil as synonymous with land-the mother- supporting and nourishing all life on the earth (Agarwal, 1967).

MATERIAL AND METHODS:

The preset study was carried out with the objective to evaluate the effect of inorganic fertilizers and organic manure on seed germination and growth behavior in Brinjal cultivar BR 112. Following treatments were used for the study:

Control (Only Soil)	:	S1
Soil + NPK	:	S2
Soil + Cow Dung	:	S3

50 replicates for each treatment were used for the study. Some important descriptions of the layout are given below:

Total number of poly bags used in the experiment	:	150
Number of poly bags used for each treatment	:	50
NPK used in 50 poly bags (grm.)	:	14.07
Cow dung (kg.) used 50 poly bags	:	25

The NPK complex fertilizer was used before sowing the seeds in S2 treatment. Cow dung meshed thoroughly for S3 treatment and mixed with soil before sowing the seeds. Total numbers of germinated plants were counted from each poly bag of all treatments at interval period of 5 days after sowing and reported as emergence count /poly bag. Plant growths were observed with different parameters i.e. plant height, number of leaves, length and width of leaves and root length. Ten normal seedlings were randomly taken at the end of the germination count for the study of plant height (shoot length), length and width of leaves were measured in cm. The no. of leaves was counted after 20 days of germination. Three plant of each treatment were randomly selected to measure the root length, which were already used to measure the other growth parameter, and the mean values were arrived at different growth stages.

RESULT:

Germination Study: The germination was influenced by different treatments. Result shows that the maximum number of seedling emergence was in S3 treatment, which contains Cow dung, in contrast to followed by S1 and S2 (Table-1).

Growth Study:

Plant height: Maximum plant height was recorded in S3 treatment, at 35 days after sowing which are higher then S1 and S2 treatment.

Number of leaves per plant: Number of leaves was recorded higher in S3 treatment in contras to S1, and S2 treatment (at 35 days after sowing).

Length & Width of leaves per plant: The leaf length and width were recorded up to 35 days after sowing of seeds in all the treatments. The length and width of green active leaves in S3 treatment is much higher then S1 and S2 treatment.

Root length: Three plants of each treatment were randomly selected to measure the root length, which were already used for other growth parameter. The root length in S3 treatment was recorded higher then all other treatments i.e. S1 and S2.

DISCUSSION:

The result of this investigation shows that the organic manure is better then the inorganic fertilizers i.e. Nitrogen, Phosphorus & Potassium, was started since "Green Revolution" to increase the yield of the crops. Day by day the use of these fertilizers was increased rapidly, in all agricultural sectors/ field of India. Farmers was used these inorganic fertilizers in more quantity to increase the yield and economy, now the use of these fertilizers is 6 to 8 times more then that time. The excess amount of the fertilizers, affect the soil as well as the crop characteristics and the product from the crop is also influenced. Fertilizers, pesticides, chemicals, etc. all contributes towards soil pollution.

The main advantages of organic manure are that it doesn't pollute the soil and not give any negative effect to environment. Because of the use of manures, the physical conditions such as aeration and water transmission properties of soil are improved. Because of the slow releases of ammoniac nitrogen and slow conversion to nitrates, the leaching loss of nitrogen is low in the presence of organic manures. The preparation of organic manure provides a hygienic and useful way of disposal and utilization of waste which wood otherwise have created and health hazards. A research was done by the agricultural chemistry's scientists of Chandrasekhar Ajad Agriculture and Technical University, Kanpur, in which it was described that the uses of inorganic fertilizers is increases day by day and by result of this the crops as well as its product, contain toxicity. According to scientists urea and other inorganic fertilizers are used in crops, because of this crop grains also contains these chemicals about 1 ppm. Dr. B. R. Gupta said that in year 1970, 18 kg/hac inorganic fertilizers were used but today it increases about 129 kg/hac. It is found that by consuming these products, which contain chemicals, are harmful for human heart, lever, stomach and kidney.

In this investigation on *Solanum melongena* L. under environmental conditions, growth and germination was evaluated, with the use of organic manure and inorganic fertilizers as a result it is found that organic manure gave better result then inorganic fertilizers.

Table: 1 Effect of different treatments on germination:

Treatments	Germination counts (%)			
	1-5 DAS	**5-10 DAS**	**10-15 DAS**	**Total**
S1	0	4	54	**58**
S2	0	18	52	**70**
S3	6	70	22	**98**

Table: 2 Effect of different treatment on Seedling growth:

S.N	Treatment	Plant height	No. of leaves	Length of leaves	Width of leaves	Root length
1.	S1	13.2 ±0.4	3.7 ±0.5	3.53 ±0.4	2.72 ±0.5	7.05 ±0.6
2.	S2	16.9 ±0.2	3.4 ±0.3	4.15 ±0.5	3.18 ±0.6	17.76 ±0.7
3.	S3	22.5 ±0.5	5.8 ±0.2	7.82 ±0.5	5.73 ±0.5	19.63 ±0.6

REFERENCE:

Biswas, T. D. & S. K. Mukharjee,Textbook of soil Science (Second edd.) (1994), Tata McGraw-Hill Publishing Company Ltd. New Delhi.

Thamburaj, S & Narendra Singh, Vegetables, Tuber Crops and Spices, (2003), Indian Council of Agricultural Research (ICAR), New Delhi.

Hartmann, H. T., D. E. Kester, F.T. Jr. davis & R. L. geneve, Plant propagation, principle & practices, (six edd.) (1992).

Handbook for Seedling Evaluation, published by, International seed Testing Association (ISTA), Zurich, Swizerland.

Seed Research, (December, 2003), A Journal devoted to the cause of seed science, Indian Society of Seed Technology, Indian Agricultural Research Institute (IARI), New Delhi.

Agrawal, R. R., Soil Fertility in India (1967), Asia publishing House, New Delhi.

Singh, S. P, Production Technology of Vegetable crops, (1989), Agricultural Res. Comm. Center, Karnal.

Tandon, H. L. S., Fertilizer management in food crops, (1993), fertilizers Development and Consultation Organization, New Delhi.

Black, C. A., Soil- plant relationship (1957), Newyork, Johan willy and sons. Inc., Newyork.

Dutt, A. K., Manures and Fertilizers, (1966), Handbook of Agriculture (ICAR), New Delhi.

Sharma, P. D., Ecology and Environment (Seventh Edd.) (2003), Rastogi Publication, Meerut, India.

3/27/2009

Fungal Diversity of Pichavaram Mangroves, Southeast Coast of India

Kandikere R. Sridhar
Microbiology and Biotechnology, Department of Biosciences, Mangalore University,
Mangalagangotri, Mangalore 574 199, Karnataka, India
E-mail: sirikr@yahoo.com

Abstract: This study reports the occurrence of fungi based on a pilot study and a checklist of fungi in Pichavaram mangroves of the southeast coast of India. Damp incubation of wood, root and leaf litter of *Avicennia marina* and *Rhizophora mucronata* yielded 15 fungi. The fungal richness was highest (9 spp.) on woody litter of both plant species and root litter of *R. mucronata*. *Aniptodera chesapeakensis*, *Halorosellinia oceanica*, *Halosarpheia marina*, *Periconia prolifica* and *Phoma* sp. were dominant (5-6.3%). Woody litter of *A. marina* was highly colonized by *H. marina* (28%), while *R. mucronata* by *Phoma* sp. (24%). The average fungi per sample ranged between 0.3 and 0.8 with a highest on woody litter (0.7-0.8). In Pichavaram mangroves, so far about 10 niches (water, sediment and live/dead plant parts) and 14 mangrove plant species have been surveyed for mycoflora. About 102 fungi consisting of mitosporic fungi (57 spp.), ascomycetes (37 spp.), phycomycetes (7 spp.) and basidiomycete (1 sp.) have been reported. Woody litter yielded a highest of 36 saprophytic fungi followed by 33 fungi as foliar epiphytes. *Cirrenalia pygmea* was the most dominant fungus, followed by *H. oceanica, P. prolifica*, *Zalerion maritima* and *Z. varia*. The foliar endophyte, *Sporormiella minima* colonized the highest number of mangrove plant species. [Nature and Science. 2009;7(5):67-75]. (ISSN: 1545-0740).

Keywords: Diversity, endophytes, epiphytes, fungi, mangroves, Pichavaram, plant detritus, saprophytes, sediment, water

Introduction

Among the tropical marine ecosystems, mangroves and coral reefs are the major habiats in gross productivity (Qasim & Wafer, 1990). Mangrove forests are spread over 181,000 km^2 in 112 countries of tropics and sub-tropics (Bunt, 1992; Spalding, 1997). Mangroves through deritus production and decomposition support a variety of planktonic, benthic and fish communities (Robertson *et al*., 1992; Alongi *et al*., 1999; Kathiresan & Bingham, 2001). Up to 41% of mangroves exist in South and South East Asia (Spalding, 1997). Indian subcontinent ranks fourth (6,700 km^2) after Indonesia, Bangladesh and Malaysia in mangrove vegetation cover (Blasco & Aizpuru, 1997). Mangrove forests in peninsular India mainly distributed in the backwater-estuarine west coast (Arabian Sea) and deltaic east coast (Bay of Bengal) (Natarajan, 1998; Kathiresan, 1999).

The Pichavaram mangroves of east coast of India have been extensively investigated for biodiversity (Kathiresan, 2000; Kumar, 2000; Kathiresan & Bingham, 2001). In 1980, it has been declared as a forest reserve and managed by the Department of Forests, Tamil Nadu (Blasco & Aizpuru, 1997; Kathiresan, 2000). Pichavaram mangroves encompasses 13 mangrove tree species (dominant: *Avicennia marina*, *Rhizophora apiculata* and *R. mucronata*) and 80 mangrove associates (trees, 24 spp.; shrubs, 21 spp.; herbs, 28 spp.; climbers, 7 spp.) (Kathiresan, 2000). According to Kathiresan (2000), the biota reported in Pichavaram mangroves include bacteria (52 spp.), fungi (23 spp.), phytoplankton (82 spp.), zooplankton (95 spp.), seagrasses (3 spp.), seaweeds (22 spp.), meiobanthos (40 spp.), macrobenthos (52 spp.), fishes (177 spp.) and birds (200 spp.). The annual plant litter production by *Rhizophora mucronata*, *R. apiculata* and *Avicennia marina* was up to 624, 1361 and 1456 g/m^2 respectively and leaf litter constitutes a major proportion (63.2-86.7%) (Muniyandi, 1986). Krishnamoorthy *et al*. (1995) indicated that up to 75% of the green cover and 90% of the forest area of Pichavaram mangroves have been lost due to human interference. Being detritus-driven ecsystem, mangroves are dependent on fungal communities for detritus mineralization and energy transfer to higher trophic levels. The aim of the present study was to investigate fungi associated with dead litter (wood, root and leaf) of two major mangrove plant species (*A. marina* and *R. mucronata*) of Pichavaram mangroves based on a pilot study and to document the mycoflora reported so far. As baseline information, the checklist facilitates comparison of fungal richness in different niches and hosts of Pichavaram mangroves with other mangroves, and to develop future strategies of conservation and exploitation of fungal diversity.

Materials and methods

Pichavaram mangroves situated at the southeast coast of India (11°29′ N, 79°46′ E) with semidiurnal tides ranging between 0.5 and 1.0 m. Freshwater enters the mangrove ecosystem through the Coleroon River and Khan Saheb Channel, while the neritic water from the Bay of Bengal thorough the Vellar River. Two stations of Pichavaram mangroves (Kidavu and Periyakadavu) have been selected to sample the mangrove detritus. The Kidavu station is situated opposite to the tourist boat jetty, while Periyakadavu at the central region of the Pichavaram mangroves. Dead and partially decomposed wood, root and leaf litter of *Avicennia marina* and *Rhizophora mucronata* accumulated on the floor were sampled during June 2007. The samples were transported to the laboratory in sterile airtight polythene bags. After sorting out the samples, attached debris was removed and 50 samples each of wood, root and leaf litter per plant species were separately incubated (25±2°C) up to six months on sterile sand-bed wetted with 50% sterile seawater in polythene bags. Once in two weeks, the samples were examined for fungal structures and the fungi grown on the substrates were identified based on the descriptions and monographs (Kohlmeyer & Volkmann-Kohlmeyer 1991; Hyde & Sarma 2000). The percent frequency of occurrence of fungi on each substrate and on all substrates were calculated:

Frequency of occurrence (%) = [(Number of samples colonized by fungal taxon) ÷ (Total number samples assessed)] × 100

Results and discussion

Pattern of fungal occurrence on damp incubated wood, root and leaf litter of *A. marina* and *R. mucronata* has been presented in Table 1. A total of 15 species (range, 6-9) of fungi (8 ascomycetes, 6 mitosporic fungi and 1 basidiomycete) was recorded. A highest of nine species was found on woody litter of both plant species and on the root litter of *R. mucronata*. The dominant fungi were: *Aniptodera chesapeakensis, Halorosellinia oceanica, Halosarpheia marina, Periconia prolifeica* and *Phoma* sp. (total frequency, 5-6.3%). The woody litter of *A. marina* was highly colonized by *H. marina* (28%), while woody litter of *R. mucronata* by *Phoma* sp. (24%). The average fungi per sample was highest on woody litter (*A. marina*, 0.8; *R. mucronata*, 0.7) than other substrates (0.3-0.4). This value is lower than other studies on woody litter of the west coast mangroves (mixed wood: 1, Borse, 1988; *Avicennia*, 2.6-2.9; *Rhizophora*, 2-2.4, Maria & Sridhar, 2003). The average fungi per wood may increase by carrying out long-term studies or more number of woody substrates. In addition, the fungal richness on woody litter depends on the difference in wood texture (hard, medium and soft), presence or absence of bark and substratum or host recurrence (Hyde *et al*., 1998; Maria & Sridhar, 2003).

Table 1. Frequency of occurrence (%) of fungi recovered from dead litter of the Pichavaram mangroves of east coast of India (*, Pneumatophores; **, Prop roots; TFO, Total frequecny of occurrence)

Fungus	*Avicennia marina*			*Rhizophora mucronata*			TFO
	Wood	Root*	Leaf	Wood	Root**	Leaf	(%)
Ascomycetes							
Halosarpheia marina (Cribb & J.W. Cribb) Kohlm.	28	6	-	-	4	-	6.3
Aniptodera chesapeakensis Shearer & M.A. Mill.	12	-	-	12	6	-	5.0
Halorosellinia oceanica (S. Schatz) Whalley, E.B.G. Jones, K.D. Hyde & Laessøe	-	2	4	8	4	12	5.0
Littispora abonnis (Kohlm.) J. Campb., J.L. Anderson & Shearer	8	-	-	-	8	-	2.7
Didymosphaeria sp.	-	8	2	6	2	2	1.3
Lindra sp.	2	4	-	2	-	-	1.3
Lulworthia sp. 1 (150 × 2.5 μm)	4	-	-	2	-	-	1.0
Lulworthia sp. 2 (250 × 2.5 μm)	4	2	-	-	-	-	1.0
Basidiomycete							
Halocyphina villosa Kohlm. & E. Kohlm.	-	4	-	2	-	-	1.0
Mitosporic fungi							
Phoma sp.	-	-	4	24	-	8	6.0
Periconia prolifica Anastasiou	-	6	10	4	8	2	5.0
Cladosporium sp.	8	-	-	10	4	4	4.3

Zalerion varia Anastasiou	-	8	2	-	2	10	3.7
Zalerion maritima (Linder) Anastasiou	6	-	8	-	-	4	3.0
Cirrenalia pygmea Kohlm.	4	-	-	-	6	2	2.0
Total fungi	**9**	**8**	**6**	**9**	**9**	**8**	
Average fungi per sample	**0.8**	**0.4**	**0.3**	**0.7**	**0.4**	**0.4**	

Table 2 shows the list of fungi in Pichavaram mangroves based on literature survey. Ten mangrove niches have been investigated for fungi: water, sediment, live (root, leaf, bark) and dead (wood, root, seedling and leaf litter) plant parts. Up to 14 mangrove hosts have been studied (*Aegiceras, Acanthus, Arthrocnemum, Avicennia* spp., *Bruguiera, Ceriops, Excoecaria, Lumnitzera, Rhizophora* spp. *Suaeda, Sesuvium* and some unidentified hosts). A total of 102 fungi (range, 3-36 species/niche) encompassing mitosporic fungi (57 spp.), ascomycetes (37 spp.), phycomycetes (7 spp.) and basidiomycete (1 sp.) have been recovered. Dead wood samples yielded the highest of 36 saprophytes (ascomycetes, 24 spp.; mitosporic fungi 11 spp., basidiomycete, 1 sp.) followed by 33 epiphytes on leaves (mitosporic fungi, 26 spp.; phycomycetes, 4 spp.; ascomycetes, 3 spp.), 21 endophytes on bark (ascomycetes, 13 spp.; mitosporic fungi, 8 spp.), 20 saprophytes on root (ascomycetes, 15 spp.; mitosporic fungi 4 spp., basidiomycete, 1 sp.) and 19 saprophytes on leaf litter (mitosporic fungi, 13 spp.; ascomycetes, 6 spp.). *Cirrenalia pygmea* was found on a maximum of six substrates, followed by *Halorosellinia oceanica* and *Periconia prolifica* on five substrates each, *Zalerion maritima* and *Z. varia* each on four substrates. Foliar endophyte, *Sporormiella minima* was associated with the highest of nine host plant species followed by *Cladosporium cladosporioides* on five hosts.

Table 2. Checklist of fungi recorded from the Pichavaram mangroves of east coast of India (references in parenthesis)
Reference: **(1)** Salique *et al*. 1985; **(2)** Venkatesan & Natarajan 1985; **(3)** Sivakumar & Kathiresan, 1990; **(4)** Kumaresan & Suryanarayanan 2001; **(5)** Kumaresan & Suryanarayan 2002; **(6)** Suryanarayanan & Kumaresan 2000; **(7)** Suryanaryanan *et al*. 1998; **(8)** Kumaresan *et al*., 2002; **(9)** Ravikumar & Vittal 1996; **(10)** Nambiar *et al*. 2008; **(11)** Rajendran & Kathiresan, 2007; **(12)** Present study
Substrate: Ac, *Aegiceras corniculatum*; Aci, *Acanthus ilicifolius*; Ai, *Arthrocnemum indicum*; Am, *Avicennia marina*; Ao, *Avicennia officinalis*; Bc, *Bruguiera cylindrica*; Cd, *Ceriops decandra*; Ea, *Excoecaria agallocha*; Lr, *Lumnitzera racemosa*; R, *Rhizophora* spp.; Ra, *Rhizophora apiculata*; Rm, *Rhizophora mucronata*; Sm, *Suaeda miritima*; Sp, *Sesuvium portulacastrum*; *, host not defined

Fungus	**Water (1)**	**Sedi-ment (1)**	**Live part**				**Dead part**			
			Epiphyte		**Endophyte**		**Wood (9,10,12)**	**Root (9,12)**	**Seed-ling (9)**	**Leaf (11,12)**
			Root (2)	**Leaf (3)**	**Leaf (4-8)**	**Bark (8)**				
Phycomycetes										
Absidia ramosa (Zopf) Lendn.	-	+	Ao,Rm	-	-	-	-	-	-	-
Cunninghamella elegans Lendn.	-	-	Ao	-	-	-	-	-	-	-
Mucor hiemalis Wehmer	-	+	-	-	-	-	-	-	-	-
M. lumbeus Bonord.	-	+	-	-	-	-	-	-	-	-
M. acemosus Fresen.	+	+	-	*	-	-	-	-	-	-
Rhizopus nigricans Ehrenb.	-	+	Ao,Rm	*	-	-	-	-	-	-
Syncephalastrum racemosum Cohn ex J. Schröt.	-	+	Ao	-	-	-	-	-	-	-
Ascomycetes										
Aigialus grandis Kohlm. & S. Schatz	-	-	-	-	-	R	R	R	-	-
A. mangrovei Borse	-	-	-	-	-	R	R	-	-	-
A. parvus S. Schatz & Kohlm.	-	-	-	-	-	R	R	R	-	-
Aniptodera chesapeakensis Shearer & M.A. Mill.	-	-	-	-	-	Am, Rm	Rm	-	-	-
Antennospora quadricornuta (Cribb & J.W. Cribb) T.W. Johnson	-	-	-	-	-	-	R	-	-	-
Ascocratera manglicola Kohlm.	-	-	-	-	-	R	R	-	-	-

Astrosphaeriella mangrovis (Kohlm. & Vittal) Aptroot & K.D. Hyde	-	-	-	-	-	R	R	R	-	-
Bathyascus mangrovei Ravik. & Vittal	-	-	-	-	-	-	-	R	-	-
Belizeana tuberculata Kohlm. & Volkm.-Kohlm.	-	-	-	-	-	-	R	-	-	-
Chaetomium globosum Kunze	-	-	-	-	Am,Ra, Rm	-	-	-	-	-
C. olivaceum Cooke & Ellis	-	+	Ao,Rm	-	-	-	-	-	-	-
Dactylospora haliotrepha (Kohlm. & E. Kohlm.) Hafellner	-	-	-	-	-	R	R	R	-	-
Didymella avicenniae S.D. Patil & Borse	-	-	-	-	-	-	-	R	-	-
Emericella nidulans (Eidam) Vuill.	-	-	Ao,Rm	-	-	-	-	-	-	-
Halorosellinia oceanica (S. Schatz) Whalley, E.B.G. Jones, K.D. Hyde & Laessøe	-	-	-	-	-	Rm	Am,Rm, *	Am, Rm	Am, Rm	Am,Rm
Halosphaeria cucullata (Kohlm.) Kohlm.	-	-	-	-	-	-	R	-	-	-
H. fibrosa Kohlm. & E. Kohlm.	-	-	-	-	-	-	-	-	-	Am/Ra
H. marina Cribb & J.W. Cribb) Kohlm.						Am	Am,Rm, R	Am, Rm	-	-
Heleococcum japonense Tubaki	-	-	-	-	-	-	R	-	-	-
Leptosphaeria australiensis (Cribb & J Cribb) G.C. Hughes	-	-	-	-	-	R	R	R	-	-
Lineolata rhizophorae (Kohlm. & E. Kohlm.) Kohlm. & Volkm.-Kohlm.	-	-	-	-	-	-	R	-	-	-
Littispora abonnis (Kohlm.) J. Campb., J.L. Anderson & Shearer	-	-	-	-	-	Am	Am,Rm	Rm	-	-
Lulworthia grandispora Meyers	-	-	-	-	-	-	R	R	-	-
Massarina thalassiae Kohlm. & Volkm.-Kohlm.	-	-	-	-	-	-	R	-	-	-
M. velataspora K.D. Hyde & Borse	-	-	-	-	-	-	R	R	-	-
Nais glitra J.L. Crane & Shearer	-	-	-	-	-	R	R	-	-	-
Neptunella longirostris (Cribb & J.W. Cribb) K.L. Pang & E.B.G. Jones	-	-	-	-	-	-	R	R	-	-
Ophiobolus littoralis (P. Crouan & H. Crouan) Sacc.	-	-	-	-	-	-	-	-	-	Am/Ra
Pontoporeia biturbinata (Durieu & Mont.) Kohlm.	-	-	-	-	-	-	-	-	-	Am/Ra
Quintaria lignatilis (Kohlm.) Kohlm. & Volkm.-Kohlm.	-	-	-	-	-	-	R	-	-	-
Savoryela lignicola E.B.G. Jones & R.A. Eaton	-	-	-	-	-	-	*	-	-	-
Spathulospora lanata Kohlm.	-	-	-	-	-	-	-	-	-	Am/Ra
Sporormiella grandispora S.I. Ahmed & Cain ex J.C. Krug	-	-	-	-	-	R	-	-	-	-
S. minima (Auersw.) S.I. Ahmed & Cain	-	-	-	-	Aci,Bc, Cd,Ea,Lr, Ra,Rm, Sm,Sp	-	-	-	-	Am,Ao
Swampomyces armeniacus Kohlm. & Volkm.-Kohlm.	-	-	-	-	-	-	-	R	-	-

Thielavia terricola (J.C. Gilman & E.V. Abbott) C.W. Emmons	-	-	Rm	-	-	-	-	-	-	-
Verruculina enalia (Kohlm.) Kohlm. & Volkm.-Kohlm.	-	-	-	-	-	-	R,*	R	R	-
Basidiomycete										
Halocyphina villosa Kohlm. & E. Kohlm.	-	-	-	-	-	-	Rm,R,*	Am, R	R	-
Mitosporic fungi										
Alternaria alternata (Fr.) Keissl.	-	-	-	*	Aci,Ai, Lr,Ra	-	-	-	-	-
A. alternata (Fr.) Keissl.	-	-	-	-	-	-	-	-	-	Am/Ra
A. tenuissima (Kunze) Wiltshire	-	-	-	*	-	-	-	-	-	-
Aspergillus aureolus Fennell & Raper	-	-	-	-	-	-	-	-	-	Am/Ra
A. brevipes G. Sm.	+	-	-	-	-	-	-	-	-	-
A. candidus Link	-	+	-	-	-	-	-	-	-	Am/Ra
A. chevalieri Thom & Church	-	-	-	-	-	-	-	-	-	Am/Ra
A. flavus Link	-	-	Ao,Rm	*	-	-	-	-	-	Am/Ra
A. fumigatus Fresen.	-	-	Ao,Rm	-	-	-	-	-	-	Am/Ra
A. glaucus (L.) Link	-	-	-	-	Rm	-	-	-	-	Am/Ra
A. nidulans (Eidam) G. Winter	-	+	Ao	-	-	-	-	-	-	-
A. niger Tiegh.	-	-	Ao,Rm	-	Cd,Ea	-	-	-	-	Am/Ra
A. ochraceus G. Wilh.	-	-	-	*	Ra	-	-	-	-	Am/Ra
A. oryzae (Ahlb.) E. Cohn	-	+	-	-	-	-	-	-	-	-
A. terreus Thom	+	+	Ao,Rm	-	-	-	-	-	-	-
A. wentii Wehmer	-	+	-	-	-	-	-	-	-	-
Camarosporium palliatum Kohlm. & E. Kohlm.	-	-	-	-	Ai,Sm	-	-	-	-	-
C. propinquum (Sacc.) Sacc.	-	-	-	-	Ai,Sm	-	-	-	-	-
Chaetomium globosum Kunze	-	-	-	-	Ra	Ra	-	-	-	-
Cirrenalia basiminuta Raghuk. & Zainal	-	-	-	-	-	-	R	-	-	-
C. macrocephala (Kohlm.) Meyers & R.T. Moore	-	-	-	-	-	-	R	-	-	-
C. pygmea Kohlm.	-	-	Rm	-	-	Am	Am,Rm, R,*	Rm, R	Rm	Rm
Cladosporium cladosporioides (Fresen.) G.A. de Vries	-	-	-	-	Am,Ao, Cd,Lr,Ra	-	-	-	-	-
C. oxysporum Berk. & M.A. Curtis	-	-	Ao,Rm	-	-	-	-	-	-	-
Colletotrichum gloeosporioides (Penz.) Penz. & Sacc.	-	-	-	-	Bc	-	-	-	-	-
Curvularia lunata (Wakker) Boedijn	-	-	Ao	-	Ao,Lr,Ra	-	-	-	-	-
C. oryzae Bugnic.	-	+	-	-	-	-	-	-	-	-
C. pallescens Boedijn	-	-	-	-	Am,Lr	-	-	-	-	-
C. tuberculata B.L. Jain	-	-	Ao	-	-	-	-	-	-	-
Dendryphiella salina (G.K. Sutherl.) Pugh & Nicot	-	-	-	-	-	-	*	-	-	-
Drechslera halodes (Drechsler) Subram. & B.L. Jain	-	-	-	-	Lr	-	-	-	-	-
D. hawaiiensis (Bugnic.) Subram. & B.L. Jain	-	-	-	-	Ra	-	-	-	-	-
D. incurvata (C. Bernard) M.B. Ellis	-	-	Ao	-	-	-	-	-	-	-
Epicoccum purpurascens Ehrenb.	-	-	-	-	-	R	R	-	-	-
Fusarium oxysporum E.F. Sm. & Swingle	-	-	Ao,Rm	*	-	-	-	-	-	-

F. solani (Mart.) Sacc.	-	-	Rm	-	-	-	-	-	-	-
Graphium penicillioides Corda	-	-	Rm	-	-	-	-	-	-	-
Humicola fuscoatra Traaen	-	-	Rm	*	-	-	-	-	-	-
Memnoniella echinata (Rivolta) Galloway	-	-	Rm	-	-	-	-	-	-	-
Microdochium oryzae (Hashioka & Yokogi) Samuels & I.C. Hallett	-	-	Rm	-	-	-	-	-	-	-
Monodictys levis (Wiltshire) S. Hughes	-	-	Ao,Rm	-	-	-	-	-	-	-
M. pelagica (T. Johnson) E.B.G. Jones	-	-	-	-	-	R	R	R	-	-
Paecilomyces varioti Bainier	-	-	Rm	-	-	-	-	-	-	-
Penicillium expansum Link	-	-	-	*	-	-	-	-	-	-
P. funiculosum Thom	-	-	Ao,Rm	*	-	-	-	-	-	-
P. nigricans K.M. Zalessky	-	-	Ao	-	-	-	-	-	-	-
Periconia prolifica Anastasiou	-	-	-	-	-	Rm	Am,Rm, R,*	Am, Rm	Am, Rm	Am,Rm
Polystigma rubrum (Pers.) DC.	-	-	Ao	-	-	-	-	-	-	-
Sphaeropsis cylindrospora Desm.	-	-	Ao	-	-	-	-	-	-	-
Trichocladium achrasporum (Meyers & R.T. Moore) M. Dixon	-	-	-	-	-	R	R	-	-	-
T. alopallonellum (Meyers & R.T. Moore) Kohlm. & Volkm.-Kohlm.	-	-	-	-	-	R	R	-	-	-
Trichoderma koningii Oudem.	-	-	Ao	-	-	-	-	-	-	-
T. lignorum (Tode) Harz	-	-	Ao	-	-	-	-	-	-	-
T. pseudokoningii Rifai	-	-	Ao,Rm	-	-	-	-	-	-	-
T. viride Pers.	-	-	Ao,Rm	-	-	-	-	-	-	-
Zalerion maritima (Linder) Anastasiou	-	-	-	-	-	Am	Am	-	Am, Rm	Am,Rm
Z. varia Anastasiou	-	-	-	-	-	-	Am,Rm, R	Am, Rm	Am, Rm	Am,Rm
Phycomycetes	**1**	**6**	**4**	**2**	**-**	**-**	**-**	**-**	**-**	**-**
Ascomycetes	**1**	**1**	**3**	**-**	**2**	**13**	**24**	**15**	**2**	**6**
Basidiomycetes	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**1**	**1**	**-**
Mitosporic fungi	**1**	**6**	**26**	**8**	**13**	**8**	**11**	**4**	**4**	**13**
Total fungi	**3**	**13**	**33**	**10**	**15**	**21**	**36**	**20**	**7**	**19**

Figure 1 compares the total fungi, mitosporic fungi and ascomycetes in different mangrove niches: water, sediment, plant surface (epiphytes: root + leaf), tissue interior (endophytes: leaf + bark) and detritus (wood, root, seedling and leaf litter). The highest number of fungi were epiphytic on root + leaf (38 spp.) followed by saprophytes on woody litter (36 spp.), endophytes on bark + leaf (35 spp.), saprophytes on root litter (20 spp.), saprophytes on leaf litter (19 spp.), and sediment inhabitants (13 spp.). Based on the extent of studies carried out, epiphytic, endophytic and saprophytic fungi may be comparable than those recovered from water and sediment. Clearly, epiphytic and endophytic fungi dominated than the saprophytic fungi. Epiphytic and endophytic fungi dominated by mitosporic fungi (30 spp. and 20 spp. respectively). Similarly, mitosporic fungi dominated as saprophytes on leaf litter (13 spp.). Woody litter and root litter showed the dominance of ascomycetes (24 spp. and 15 spp. respectively).

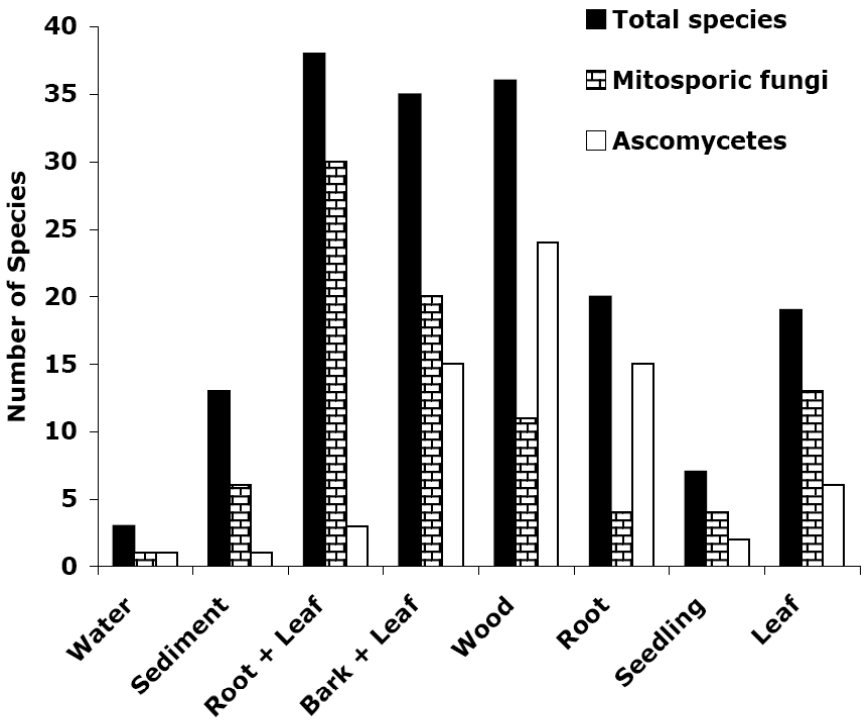


Figure 1. Fungi reported in different niches of Pichavaram mangroves: water, sediment, epiphytes (root + leaf), endophytes (bark + leaf) and saprophytes (wood, root, seedling and leaf)

So far, woody litter of *Avicennia* and *Rhizophora* yielded up to 36 species of fungi in Pichavaram mangroves (Table 2). Fungal richness on woody litter of *Avicennia* and *Rhizophora* was highest (58 spp.) in Udyavara mangrove of the west coast (Maria & Sridhar 2003). Sarma & Vittal (2001) also recovered maximum fungi on woody litter of *Rhizophora* (64 spp.) and *Avicennia* (55 spp.) from the Godavari and Krishna deltas of east coast. The nutritional features and persistent nature of woody litter of *Avicennia* and *Rhizophora* in mangrove habitats might be responsible for yielding rich mycoflora (Maria *et al*., 2006). Mycological investigations carried out in Pichavaram mangroves are relatively meager compared to biodiversity studies carried out on other flora and fauna (Kathiresan, 2000). As Pichavaram mangroves consists of 37 tree species (mangrove, 13 spp.; mangrove associates, 24 spp.) (Kathiresan, 2000), at least 1,200 fungi might exist based on plant-fungus ratio, 1:33 (Fröhlich & Hyde, 1999). The checklist reveals only 102 fungi, which accounts to about 8% of fungi exist in Pichavaram mangroves. This comparison is also applicable to other Indian mangroves as intense studies have not been carried out. Up to 2006, higher fungi of the Indain mangroves reported were 165 species (ascomycetes, 111 spp.; mitosporic fungi, 53 spp.; basidiomycete, 1 sp.) (Sridhar, 2009). Excluding phycomycetes (7 spp.), higher fungi of Pichavaram mangroves accounts to about 58% of Indian mangrove fungi. However, the chicklist of fungi of Pichavaram mangroves presented in Table 2 consists of fungi identified up to species level. There are many reports from Pichavaram mangroves listing only genera of some fungi indicating the necessity of intense study of fungal systematics.

Several mycological investigations of the Indian mangroves have been pursued or intensified after the biodiversity initiative in 1992. There seems to be no studies on the pattern of decomposition of mangrove leaf and woody litter in the east coast (Maria *et al*., 2006; Ananda *et al*., 2008). Similarly, endophytic fungal studies carried out are confined to leaves and bark, while roots are not studied (Ananda & Sridhar, 2002). Water and sediments are also not explored intensely for fungal resource. Based on overall biodiversity investigations carried out at the Pichavaram mangroves, this mangrove forest deserves to be considered as a model mangrove of the Peninsular India for comparison with other tropical and subtropical regions. First important step of conservation and sustainable use of biodiversity of Pichavaram mangroves is to elevate the status from forest reserve to a national biodiversity park. The second equally important step would be protection of Pichavaram mangroves from human interference by providing

alternative socioeconomic avenues and resources to the human population currently heavily depending on the Pichavaram mangrove habitats.

References

1. Alongi DM, Tirendi F, Dixon P, Trott LA, Brunskill GJ. Mineralization of organic matter in intertidal sediments of a tropical semi-enclosed delta. Estuarine, Coastal and Shelf Science 1999;48:451-467.
2. Ananda K, Sridhar KR. Diversity of endophytic fungi in the roots of mangrove species on the west coast of India. Canadian Journal of Microbiology 2002;48:871-878.
3. Ananda K, Sridhar KR, Raviraja NS, Baerlocher F. Breakdown of fresh and dried *Rhizophora mucronata* leaves in a mangrove of Southwest India. Wetlands Ecology and Management 2008;16:1-9.
4. Blasco F, Aizpuru M. Classification and evolution of the mangroves of India. Tropical Ecology 1997;38:357-374.
5. Borse BD. Frequency of occurrence of marine fungi from Maharashtra coast, India. Indian Journal of Marine Sciences 1988;17:165-167.
6. Bunt JS. Introduction. In: Robertson AI, Alongi DM, ed. Tropical mangrove ecosystem. American Geophysical Union, Washington DC. 1992: 1-6.
7. Fröhlich J, Hyde, KD. Biodiversity of palm fungi in the tropics: Are global fungal diversity estimates. Biodiversity and Conservation 1999;8:977-1104.
8. Hyde KD, Sarma VV. Pictorial key to higher marine fungi. In: Hyde KD, Pointing SB, ed. Marine Mycology - A Practical Approach. Fungal Diversity Press, Hong Kong. 2000: 205-270.
9. Hyde KD, Jones EBG, Leaãno E, Pointing SB, Poonyth D, Vrijmoed LLP. Role of fungi in marine ecosystems. Biodiversity and Conservation 1998; 7:1147-1161.
10. Kathiresan K. Global biodiversity of mangroves in relation to India. In: Proceedings of UNU International Training Workshop on Methodologies for Assessment of Biodiversity in Estuaries, Mangroves and Coastal Waters. Centre of Advanced Study in Marine Biology, Annamalai University, India. 1999:105-122.
11. Kathiresan K. A review of studies on Pichavaram mangrove, Southern India. Hydrobiologia 2000;430:185-205.
12. Kathiresan K, Bingham BL. Biology of mangroves and mangrove ecosystems. Advances in Marine Biology 2001;40:81-251.
13. Kohlmeyer J, Volkmann-Kohlmeyer B. Illustrated Key to the filamentous higher marine fungi. Botanica Marina 1991;34:1-61.
14. Krishnamoorthy R, Sundaramoorthy S, Mohan D, Gowri DS, Ramchandran S. Status of mangroves along Tamil Nadu coast and their special properties – A remote sensing approach. In: Muralikrishna IV, ed. Remote Sensing and Geographical Information Systems for Environmental Planing. Tata McGraw Hill Publ. Ltd., New Delhi. 1995.
15. Kumar RS. A review of biodiversity studies of soil dwelling organisms in Indian mangroves. Zoos' Print Journal 2000;15:221-227.
16. Kumaresan V, Suryanarayanan TS. Occurrence and distribution of endophytic fungi in a mangrove community. Mycological Research. 2001;105:1388-1391.
17. Kumaresan V, Suryanarayanan TS. Endophyte assemblages in young, mature and senescent leaves of *Rhizophora apiculata*: evidence for the role of endophytes in mangrove litter degradation. Fungal Diversity 2002;9:81-91.
18. Kumaresan V, Suryanarayanan TS, Johnson JA. Ecology of mangrove endophytes. In: Hyde KD, ed. Fungi of Marine Environments. Fungal Diversity Press, Hong Kong. 2002;145-166.
19. Maria GL, Sridhar KR. Diversity of filamentous fungi on woody litter of five mangrove plant species from the southwest coast of India. Fungal Diversity 2003;14:109-126.
20. Maria GL, Sridhar KR, Baerlocher F. Decomposition of dead twigs of *Avicennia officinalis* and *Rhizophora mucronata* in a mangrove in southwest India. Botanica Marina 2006;49:450-455.
21. Muniyandi K. Studies on mangroves of Pichavaram (south-east cost of India). Ph.D. thesis. Annamalai univesity, Parangipettai, India. 1986.
22. Nambiar GR, Ravindran K, Changxing Z, Jaleel CA. A glimpse of lignicolous marine fungi occurring in coastal waterbodies of Tamil Nadu (India). Comptes Rendus Biologies 2008;331:475-480.
23. Natarajan R. The mangroves: a natural, renewable economic resource needs conservation. In: An Anthology of Indian Mangroves. CAS in Marine Biology, ENVIS Centre, Annamalai University, India. 1998;1-6.

24. Qasim SZ, Wafar MVM. Marine resources in the tropics. Resource Management and Optimization 1990;7:141-169.
25. Rajendran N, Kathiresan K. Microbial flora associated with submerged mangrove leaf litter in India. Revista de Biología Tropical 2007;55:293-400.
26. Ravikumar DR, Vittal BPR. Fungal diversity on decomposing biomass of mangrove plant *Rhizophora* in Pichavaram estuary, east coast of India. Indian Journal of Marine Sciences 1996;25:142-144.
27. Robertson AI, Alongi DM, Boto KG. Food chains and carbon fluxes. In: Robertson AI, Alongi DM, ed. Tropical mangrove ecosystems. American Geophysical Union, Washington. 1992:293-329
28. Salique SM, Krishnamurthy K, Palaniappan R. Ecology of fungal population in the Vellar Estuary and Pichavaram mangroves, Southern India. In: Bhosale LJ, ed. The Mangroves. Shivaji University, Kolhapur, India. 1985:153-162.
29. Sarma VV, Vittal BPR. Biodiversity of manglicolous fungi on selected plants in the Godavari and Krishna deltas, east coast of India. Fungal Diversity 2001;6:115-130.
30. Sivakumar A, Kathiresan K. Phylloplane fungi from mangroves. Indian Journal of Microbiology 1990;30:229-231.
31. Spalding MF, Lasco F, Field C. World Mangroves Atlas. Cambridge Samara Publ Co, Cambridge. 1997.
32. Sridhar KR. Mangrove fungi of the Indian Peninsula. In: Sridhar KR, ed. Frontiers in Fungal Ecology, Diversity and Metabolites. IK International Publishing House Pvt Ltd, New Delhi. 2009:28-50.
33. Suryanarayanan TS, Kumaresan V. Endophytic fungi of some halophytes from an estuarine mangrove forest. Mycological Research 2000;104:1465-1467.
34. Suryanarayanan TS, Kumaresan V, Johnson JA. Foilar fungal endophytes from two species of the mangrove *Rhizophora*. Canadian Journal of Microbiology 1998;44:1003-1006.
35. Venkatesan T, Natarajan R. Intertidal mycoflora of Pichavaram mangroves near Porto Novo. In: Bhosale LJ, ed. The Mangroves. Shivaji University, Kolhapur, India. 1985;163-169.

3/27/2009

Bark Factors Affecting the Distribution of Epiphytic Ferns Communities

Lalit M. Tewari[1*], Geeta Tewari[2] , Tapan Nailwal [3] and Y.P.S. Pangtey[4]
[1*,4]Department of Botany, [2]Departement of Chemistry, [3]Department of Biotechnology
D.S.B. Campus, Kumaun University, Nainital-263002 (Uttarakhand), India
*Corresponding author E-mail: l_tewari@rediffmail.com

Abstract: Substratum plays an important role in the growth and behaviour of the plants. In present study 10 dominant phorophytes (host trees) were analyzed for their bark characteristics. All angiospermous bark except *Rhododendron arboreum* Smith were invariably hard and markedly rough textured while gymnospermous barks were also very hard and rough texture except *Cupressus torulosa* D. Don. All the tree barks studied found to have different range of acidity. The moisture content varied from 35.3 % (*Shorea robusta* Gartn. f.) to 120.7 % (*Cupressus torulosa* D. Don.). All the chemicals except calcium showed mixed pattern of range. Bark texture and moisture content in general are important for epiphytic ferns. The majority of epiphytic ferns were recorded from moist shady places suggesting that these ferns communities demand high humidity for their growth and survival. [Nature and Science. 2009;7(5):76-81]. (ISSN: 1545-0740).

Keywords: Substrate, epiphytic ferns, trees, bark.

1. Introduction

Although climate and geography play an important role in the distribution and composition of epiphytic communities, the texture and water relations of bark are also considered no less significant in the life of cryptogamic plants. The importance of substrate for the growth of cryptogamic vegetation has attracted considerable attention. According to Brodo (1973), the most tangible elements of a plant's environment are its substrate, the material on which the plant grows. The external texture of the substrate is also important in supporting the rhizome, in trapping the spore, capturing and retaining the moisture and chemical substances and also provides a platform for other organisms. The nature of bark and its importance in determining the composition and structure of epiphytic communities has been studied earlier by Barkman (1958), Iwatsuki (1960), Beals (1965), Smith (1982), Bates (1992),Tewari (1992), Gustafsson and Eriksson (1995), Klaus et al. (2005) and Hauck et al. (2006). Khullar (1981) also suggested that the bark of trees is a factor meriting considerable importance for the prevalence of epiphytic ferns. While Barkman (1958) extensively reviewed the literature on this subject, including such factors as bark relief, flaking or scaling, hardness, moisture holding capacity, presence of resin and tannin, salt concentration, pH and buffer capacity etc. Keeping this in view, an attempt has been made in the present study to explore the bark characteristics of dominant trees species of each dominant forest types and its possible relationship with epiphytic ferns.

2. Methodology

The bark samples of dominant trees from nine different forests : Sal forest (700-900m), Miscellaneous forest (900-1200m), Chir-pine forest (1200-1400m), Mixed chir-pine and banj-oak forest (1400-1700m), Banj-oak forest (1700-200m), Telonj-oak forest (2000-2300m), Cypress forest (2300-2500m), Deodar forest (2400-2611m) and Kharsu-oak forest (2500-2611m) were collected for chemical and physical analysis in the month of August, when the growth of epiphytic ferns was maximum. However, for pH and moisture contents, the bark samples were collected in mid June, August and September last, as premonsoon, monsoon and post monsoon samples. The various parameters studied were: the texture, colour, water or moisture holding capacity, moisture content and pH. The nutrient contents (organic C, N, Ca, P, K) of bark samples were also chemically analyzed.

For each parameter, three samples were taken. However, texture, colour, hardness and softness of bark were noted by visual observation during the field survey.

pH was measured by using pH meter and moisture content and moisture holding capacity were expressed as the ratio of water absorbed to dry weight of sample. Organic carbon content was determined by Walkely and Black method (1934). Calcium and exchangeable magnesium were determined by digestion and gravimetrical method given by Pipper (1944). For the determination of available phosphorus, spectropphotometre and for potassium, flame photometry method of Jackson (1958) was used. The physical and chemical properties of bark are presented in Table 1 and 2 respectively for dominant tree of each forest.

In all, 25 species of epiphytic ferns were collected and identified belonging to 15 genera and 6 families. The various species dealt in the present study include: *Araiostegia pseudocystopteris* (Kunze) Copel., *Arthromeris wallichiana (*Spreng.) Ching , *Asplenium ensiforme* Wall. Ex Hook. *et* Ching, *A. indicum* Sledge, *Drynaria mollis* Bedd., *D. propinqua (*Wall. Ex Mett.) J. Smith, *Lepisorus kashyapii* (Mehra) Mehra, *L. Nudus* (Hook.) Ching, *L. pseudonudus* Ching, *L. scolopendrium* (Buch. –Ham. Ex D. Don) Mehra *et* Bir, *L. tenuipes* Ching *et* Kullar, *Leucostegia immersa* Ching *et* Kullar, *Loxogramme involuta* (D.Don) Presl, *Microsorum membranaceum* (D.Don) Ching, *Oleandra wallichii* (Hook.) Presl, *Paradavallodes membranulosum* (Wall. Ex Hook.) Ching, *Phymatopteris oxyloba* (Wall. Ex Kunze) Pichi Sermolli, *Polypodiastrum argutum* (Wall. Ex Hook.) Ching, *Polypodiodes amoena* (Wall. Ex Mett.) Ching, *P. lachnopus* (Wall. Ex Hook.) Ching, *P. microrhizoma* (Clarke ex Baker) Ching, *Pyrrosia flocculosa* D. Don) Ching, *P. mannii* (Gies.) Ching, *P. stictica* (Kunze) Holtt. and *Vittaria flexuosa* Fe'e.

3. Results and discussion

In present study, ten dominant phorophytes (host trees) were analysed for their bark characteristics viz. *Shorea robusta* Gartn. f., *Boehmeria rugulosa* Wedd., *Toona ciliata* Roem., *Pinus roxburghii* Sarg., *Quercus incana* Roxb., *Q. floribunda* Rehder, *Q. semecarpifolia* Smith, *Rhododendron arboreum* Smith, *Cupressus torulosa* D. Don and *Cedrus deodara* Loud.

3.1. Physical nature of bark

In the present study, the bark of an agiospermous tree i.e. oak was mainly dark reddish brown to light grey or blackish in colour and very rough in texture. Regarding the hardness, all the trees had hard natured barks. On the other hand, gymnospermous tree barks varied in colour. In *Pinus roxburghii* Sarg., the bark colour varied from grey to pinkish-brown, smooth and hard in nature, while the colour of *Cedrus deodara* Loud. bark was brown to reddish, rough and hard in nature. Contrary to this, *Cupressus torulosa* D. Don bark had remarkably different features i.e. the colour of the bark was pale of dark brown-reddish and soft in nature exfoliating in fibrous strips. In general, gymnospermous trees had soft textured barks compared to angiospermous tree barks (Table 1).

3.2. Moisture content

The moisture content of the bark depends upon bark texture (Smith 1982). The moisture content was recorded maximum during monsoon period (August) in all the trees barks studied and it varied from 35.3% (*Shorea robusta* Gartn. f.) to 120.7% (*Cupressus torulosa* D. Don). Among gymnospermous bark, moisture content was maximum (120.7%) in *Cupressus torulosa* D. Don and minimum (80.2%) in *Cedrus deodara* Loud. during August. In angiospermous bark, it was maximum (70.2%) in *Quercus floribunda* Rehder and minimum (36.3%) in *Shorea Robusta* Gartn. f. during August (Table 1).

3.3. Moisture holding capacity

Gilbert (1970) concluded that water supply was independent for substrate. Billing and Dew (1938) did found that hemlock bark both a lower water absorption capacity and higher water loss than did tulip poplar bark and they also showed that the absorptive capacity of bark changed with height, exposure and epiphytic cover. In general, water-holding capacity of barks varied from 38.2% (*Shorea robusta* Gartn. f.) to 126.2% (*Rhododendron arboreum* Smith) in all trees studied. It also indicated that the gymnospermous trees had greater moisture holding capacity compared to angiospermic trees. Exceptionally maximum moisture holding capacity was recorded in *Rhododendron arboreum* Smith bark. Among gymnospermous tree barks, water-holding capacity was maximum in *Cupressus torulosa* D. Don (125%) and minimum in *Pinus roxburghii* Sarg. (85.2%), while other angiospermous tree barks had comparatively low moisture holding capacity i.e. *Quercus floribunda* Rehder had 72.2% (maximum), while *Shorea robusta* Gartn. f. 38.2%(minimum) moisture holding capacity. The low moisture holding capacity in latter case was probably due to rough texture and hardness and since the moisture holding capacity of bark depends upon the density, porosity, texture and internal structure of bark (Table 1).

3.4. pH

A number of workers had determined the pH of bark in many trees species and emphasized that the differences in epiphytic community was due to the difference in moisture content and pH of the various barks. The pH of the bark and the epiphytic communities are closely related to bark texture and humid condition. In general, all bark types studied were acidic in nature and it was maximum during monsoon

period (August) in each case and varied between 6.6 to 4.8. Tewari (1992) also recorded maximum pH during monsoon for tree barks.

The pH of gymnospermous tree barks was comparatively more acidic than angiospermous. In gymnospermous tree bark, it was less acidic in *Cupressus torulosa* D. Don (5.9) and more acidic in *Quercus incana* Roxb. (6.6) and more acidic in *Shorea robusta* Gartn.f. (5.1) during monsoonal period. In all three barks studied, pH fluctuated in a narrow range. However, it was maximum during monsoonal period (Table 1).

Table 1. Bark characteristics, moisture content (MC), pH and moisture holding capacity (MHC) of dominant tree species

Sl. No.	Name of host tree	Bark characteristics	Months						
			June		August		September		MHC (%)
			MC (%)	pH	MC (%)	pH	MC (%)	pH	
1	*Shorea robusta* Gartn.f.	Dark reddish-brown or grey, long deep and wide vertical fissures, 1.8-3.1 cm thick	15.6	4.6	35.3	5.1	25.5	4.8	38.2
2	*Boehmeria rugulosa* Wedd.	Dark brown, rough and deeply furrowed, 2.6cm thick	20.2	6.0	43.5	6.5	28.6	6.2	42.3
3	*Toona ciliata* Roem.	Dark grey or reddish-brown, rough with shallow reticulate cracks, exfoliating in irregular woody scales, 1.3-1.7cm thick	30.3	5.9	50.5	6.5	40.6	6.1	48.0
4	*Pinus roxburghii* Sarg.	Grey or pinkish-brown, very rough and deeply fissured longitudinally, shallow cracks into irregular scales	40.2	4.4	80.6	4.8	50.2	4.5	85.2
5	*Quercus incana* Roxb.	Pale grey or dark-reddish brown, rough with shallow cracks, exfoliating in irregular woody scales, 1.3-2.6 cm thick	42.0	6.0	68.8	6.6	40.2	6.2	68.8
6	*Quercus floribunda* Rehder	Dark grey or dark reddish-brown, rough with shallow cracks, exfoliating in irregular woody scales, 1.3-2.6cm thick	40.2	5.8	70.2	6.2	48.4	5.5	72.2
7	*Rhododendron arboreum* Smith	Pinkish brown, rough, exfoliating in thick flakes, 0.5-1.3cm thick	56.3	4.8	85.2	5.4	63.0	4.8	126.2
8	*Cupressus torulosa* D. Don	Pale or dark brown-reddish, rough deep vertical fissure, exfoliating in fibrous strips, 1.3-4cm thick	59.0	5.7	120.7	5.9	76.2	6.0	125.0
9	*Cedrus deodara* Loud.	Brown often reddish, deep furrows separated by woody ridges, 3.5-5cm thick	45.6	5.2	80.2	5.6	39.2	5.3	88.3
10	*Quercus semecarpifolia* Smith	Silvery grey to blackish rough with shallow cracks, exfoliating in irregular woody scales, 1-2.6cm thick	36.4	5.8	60.4	6.1	40.6	5.8	60.2

3.5. Chemical content

It is often suggested that the chemical nature of the bark is important in determing the composition of epiphytic communities. Various workers emphasized the importance of the chemical nature of barks. Some of them are Barkman (1958) and Smith (1982). In present study, total organic carbon percent in all barks varied between 73.88 (*Quercus incana* Roxb.) to 88.84 (*Rhododendron arboreum* Smith). Gymnospermous tree barks had higher total organic content than angiospermous barks, it ranged from 83.50 (*Cedrus deodara* Loud.) to 87.18 (*Pinus roxburghii* Sarg.) (Table 2).

Among all the tree barks, total nitrogen percent varied between 0.28 (*Cedrus deodara* Loud.) to 0.50 (*Cupressus torulosa* D. Don and *Quercus semecarpifolia* Smith), while remaining species showed a mixed pattern of total nitrogen (Table 2).

The percent phosphorus varied in bark species from 0.008 (*Shorea robusta* Gartn. f.) to 0.55 (*Cedrus deodara* Loud.). The content of percent potassium varied from 0.10 (*Quercus incana* Roxb.) to 0.17 (*Quercus semecarpifolia* Smith), while the percent magnesium ranged from 0.011 (*Rhododendron arboreum* Smith) to 0.020 (*Quercus incana* Roxb.) (Table 2).

On the other hand, all the tree barks were fairly rich in calcium content. Percent calcium ranged from 1.90 (*Cupressus torulosa* D. Don) to 6.10 (*Rhododendron arboreum* Smith). In general, the angiospernous tree barks had higher calcium content in comparison to the gymnosperms. In gymnospermous tree barks, it ranged from 1.90 (*Cupressus torulosa* D. Don) to 2.60 (*Cedrus deodara* Loud.) while 2.60 (*Shorea robusta* Gartn. f.) to 6.10 (*Rhododendron arboreum* Smith) in angiospermous trees (Table 2).

Table 2. Chemical content of bark of dominant tree species

C= Organic carbon, N= Nitrogen, P= Phosphorous, K= Potassium, Ca= Calcium, Mg=Magnisium.

Sl. No.	Name of host tree	Chemical contents in percent					
		C	N	P	K	Ca	Mg
1	*Shorea robusta* Gartn. f.	86.21	0.30	0.008	0.04	2.60	0.013
2	*Boehmeria rugulosa* Wedd.	74.82	0.37	0.009	0.05	2.92	0.016
3	*Toona ciliata* Roem.	76.81	0.40	0.010	0.05	3.21	0.016
4	*Pinus roxburghii* Sarg.	87.18	0.28	0.007	0.03	2.30	0.012
5	*Quercus incana* Roxb.	73.88	0.40	0.021	0.10	5.70	0.020
6	*Quercus floribunda* Rehder	75.88	0.44	0.012	0.06	4.80	0.019
7	*Rhododendron arboreum* Smith	88.84	0.34	0.014	0.02	6.10	0.011
8	*Cupressus torulosa D. Don*	83.54	0.50	0.017	0.06	1.90	0.015
9	*Cedrus deodara* Loud.	83.50	0.28	0.055	0.06	2.60	0.017
10	*Quercus semecarpifolia* Smith	78.92	0.50	0.018	0.17	4.30	0.016

3.6. Epiphytic fern diversity

Rhododendron arboreum Smith displayed high species richness of ferns (15 species), followed by *Quercus incana* Roxb. (12 species) and *Q. floribunda* Rehder (12 species). Besides, a high species richness was also noticed on *Pinus roxburghii* Sarg. (8 spp.), *Cedrus deodara* Loud. (7 spp.), *Toona ciliata* Roem. (9 spp.), *Boehmeria rugulosa* Wedd. (7 spp.), *Cupressus torulosa* D. Don (6 spp.) and *Quercus samecarpifolia* Smith (4 spp.). However, poor richness of ferns was displayed by *Shorea robusta* Gartn. f. on which only two species were recorded. While in case of *Cupressus torulosa* D. Don, trees restricted to certain localities of forest i.e. forest marginal trees, supported epiphytic ferns but in general their growth was rather poor and scanty.

Earlier, a few workers have emphasized that generally the gymnospermous trees lack epiphytic ferns (Mehra 1939, Dhir, 1980). In contrast to general belief that epiphytic ferns shun coniferous bark, a rich ferns growth on a number of gymnospermous trees vis. *Pinus roxburghii* Sarg. and *Cedrus deodara*

Loud. was observed. Between 2400-2611m the *Cedrus deodara* Loud. tree supported an abundant growth of *Lepisorus kashyapii* (Mehra) Mehra and *Drynaria mollis* Bedd.. Similarly, at certain localities at lower altitudes, the chir pine (*Pinus roxburghii* Sarg.) trees were also frequently inhabited by the ferns. This indicates that the ferns may grow and flourish on any host including gymnosperms, if the conditions are favourable.

The substratum is the most tangible element of plant environment, on or in which the plant grow. Culberson (1955) found that there was no direct correlation between epiphytic communities and hardness of bark. Khullar (1981) suggested that the trees with cracked up (spongy) barks are generally more suitable for epiphytic fern growth. This statement seems to be correct because in case of tree saplings and many shrubs, the poor representation of epiphytic ferns were observed and this may be due to their compact and intact barks. *Cupressus torulosa* D. Don also had poor growth of epiphytic ferns owing to its nature of bark. While *Rhododendron arboreum* Smith had smooth and spongy bark, which accumulate higher humus and thus rendering it most suitable host for the growth and prevalence of epiphytic ferns. Martin (1938) and Iwatsuki (1960) suggested that the chemical nature of bark is important in determining the composition of epiphytic communities. All the chemicals except calcium showed mixed pattern of range. Calcium percent was higher in angiospermous tree barks compared to gymnospermous tree barks.

Majority of epiphytic ferns (*Aplenium ensiforme* Wall. Ex Hook. *et* Ching, *Oleandra wallichii* (Hook.) Presl, *Loxogramme involuta* (D.Don) Presl , *Leucostegia immersa* Ching *et* Kullar, *Microsoum membranaceum* (D.Don) Ching and *Vittaria flexuosa* Fe'e*)* were recorded from moist-shady places suggesting that these ferns demand high humidity and require very little light for their growth and survival. However, many of them like *Lepisorus nudus* (Hook.) Ching, *Pyrrosia* species and *Drynaria mollis* Bedd.etc. have well adapted for open and exposed conditions due to their thick leathery, hairy and sterile winter fronds., while *Araiostegia pseudocystopteris* (Kunze) Copel shows a wide range of distribution on different habitats.

Colour, texture and exfoliation rate of gymnospermous and angiospermous bark were of immense value regarding the presence or absence of the epiphytic vegetation. Trees with a cracked up (spongy) bark are generally suitable for epiphytic fern communities.

4. Conclusion

On the basis of the study, it can be concluded that only bark texture and moisture content are important for epiphytic fern communities. During monsoon, the humidity remains fairly high, thus epiphytic ferns attain their maximum development and complete their life cycle before the advent of early winter. The luxuriance of epiphytic ferns not only on angiospermous trees but even the gymnospermous trees has epiphytes on them. The climate and geography do play an important role in deterring the make up of epiphytic communities.

Acknowledgement

The authors are thankful to Prof. C.P. Barthwal, Vice Chancellor, Prof. R.C.Pant, Ex. Vice Chancellor, Prof. K.N. Pandey, Dean Science, Prof. C.S. Mathela, Head Chemistry and Prof. Uma Palni, Head Botany for encouragement and providing facilities. The financial assistance provided by Department of Biotechnology, New Delhi and Uttarakhand Council of Science and Technology, Dehradun is greatly acknowledged.

References

Barkman, J.J. Phytosociology and Ecology of Cryptogamic Epiphytes. Van Gorcum, Assen. Netherland, 1958.
Bates, J. W. *J. Ecol.* 1992; **80**:163-179.
Beals, E.W. *Oikos* 1965;16:1-8.
Billing, W. D. Drew, W. B. *Amer. Midl. Natur* 1938;20:302-330.
Brodo, I.N. The lichens, Academic Press, New york 1973: 401-441.
Culberson, W.L. *Ecological* Monographs 1955;25:215-231.
Dhir, K.K. *Bibliotheca Pteridologia* 1980;1:1-158.
Gilberts, O.I. *New Phytologist* 1970;69:605-627.
Gustafsson L., Eriksson I. *Journal of applied ecology* 1995; 32(2): 412-424.
Hauck, Marcus, Hoffman, Erika, Schmull, Michaela. *Ann. Bot. Fennici*2006; 43:1-12.
Iwatsuki, Z. *Jour. Hattori. Bot. Lab.* 1960;22:159-350.

Jackson, M.L. Soil Chemical Analysis, Prentice Hall Inc. Engle Woodcliffs. N.J. USA. 1958:1-498.
Khullar, S.P. Contemprary Trends in Plant Sciences Kalyani Publication, New Delhi. 1981:202-207.
Klaus, Mehltreter, Alejandra, Flores-Palacios, José G, Garcia-Franco. *Journal of tropical ecology* 2005; 21 (6): 651-660.
Martin, N.M. *J.Ecol.* 1938; 26:82-95.
Mehra, P.N. Ferns of Massuri P.U.Publ. Lahaur. 1939:1-59.
Pipper, C.S. soil and plant analysis Interspecies Publication Inc, New York.1944:1-368.
Smith, A.J.E. Bryophite Ecology Chapman and Hall, London 1982:191-227.
Tewari, L.M. Ph.D. Thesis Kumaun University, Nainital, 1992.
Walkey, K.E. Black, J.A. *Soil Science* 1934;37:29.

4/4/2009

Soyabean Seed Quality Evaluation

Sunil Chandra Joshi*, N. Indrakumar Singh** and J.S. Chauhan***

*M. Phil. Student, Global Open University, Nagaland (India). E-mail: cool.suniljoshi@rediffmail.com
**Research Scholar, Department of Seed Science & Technology, H.N.B. Garhwal University, Srinagar Garhwal, (Uk)-246 174 (India). E. mail: indrasst08@gmail.com
***Assoc. Prof. & Head, Department of Seed Science & Technology, H.N.B. Garhwal University, Srinagar Garhwal, (Uk)-246 174 (India). E. mail: jai002004@yahoo.com

Abstract: Seed quality is very essential for optimum stand establishment and maximum yield potential in soyabean. As a result, it is necessary to have different seed testing parameters that permit rapid, objective and accurate evaluation of seed quality. This investigation evaluated physical and physiological seed quality components of four seed lots of one soyabean (*Glycine max* L. Merr.) cultivar PS-1092 collected from different seed farms of Almora district of Uttarakhand (India). Each sample was divided into four replicates. Seed quality was evaluated by physical purity, standard germination, imbibition rate, seed vigour test and pure live seed. Experimental results has shown that, sample S2 recorded the maximum pure live seed (95.06 %) with high vigour and sample S3 showed the minimum pure live seed with 79.54%. So, as per observation recorded, sample S_2 and S_4 meets the minimum standard of purity and germination percent. In another case purity percent of sample S_1 and S_3 was below the minimum standard but the germination percent was very high from the recommended standard. So from the discussion it may be concluded that as seed lot 2 (Sample S2) possessed the highest quality than any other seed lots but all the remaining three seed lots were found good enough to use as a planting material in the next season as the quality value was within the standard value. [Nature and Science. 2009;7(5):82-85]. (ISSN: 1545-0740).

Key Words: Quality, germination, purity, vigour, planting value

Introduction

Soyabean seed evaluation of vigour and its relation with field seedling emergence can provide secure indicatives of seed physiological potential. The production of high quality seed results in many direct benefits to the seed consumer and seed producer. Use of high quality seed affords the seed consumer many production options; because high quality seeds better tolerate stressful planting conditions. Planting a superior seed lot usually results in a more uniform stand that allows better secondary tillage and weed/pest control. All of these factors promote earlier and more uniform emergences, which can lead to increase yield and more economic return to seed consumer.

Different seed testing parameter's results will provide the basic seed quality information and aid in planting decisions. Growers should conduct germination test immediately after harvest to determine if the seed is worth saving and again before planting to see if they are worth planting. Seed quality can change dramatically during storage, so testing twice is always good. The quality of the seed lot is judged by the relative percentage of various components. The quality is considered superior, if pure seed percentage is above 98, and other seeds and inert matter percentage as low as possible. The purity test is done with the object of determining the composition by weight of the sample being tested, and by inference the composition of the seed weight. Since germination test are based on pure seed components, it can readily be seen that purity analysis and germination tests compliment each other. Thus the actual planting value of seed can be determined only when the purity analysis and germination tests are considered together. One of the primary factors is access to moisture for the seed

(McDonald & Copeland, 2004; Hartmann and Kester, 1999). High physiological potential (germination and vigour) of a given seed lot credentialed it for a superior performance in a broad range of field environmental conditions (Egli & TeKrony, 1996; Marcos Filho, 1999).

The investigation was conducted to characterize the quality of soyabean seeds by comparing the different testing parameters with respect to the Indian Minimum Seed Standard in order to maintain the quality of the seed for further generation.

Materials and Methods

The investigation was conducted at the Seed Testing Laboratory of Dept. of Seed Science and Technology, H.N.B. Garhwal University. According to the Indian Minimum Seed Standard the germination percent of soyabean is 70 % and purity percent is 98 %.

Four samples of soyabean (*Glycine max* L. Merr.) cultivar PS-1092 seeds were collected from different seeds lots of different villages of Almora district of Uttarakhand (India). Each sample was assigned as S1, S2, S3 and S4 and divided into four replicates for each sample. The work consists of purity test, standard germination test, imbibition rate, seed vigour test and pre live seed.

Purity analysis sorted out three components; inert matter, other seed and pure seed. The three components were weighed by using the Electronic Balance having the accuracy of $\pm$ 0.001g and expressed in percentage. Standard germination test was conducted on a 100 seeds per replicate at 25^{0}C for six days in germinator by using filter paper and, for seedling emergence test sand was taken as substratum and kept at the same temperature. Pure live seed (PLS) percentage represents the amount of pure seeds in a seed lot that are capable of producing seedlings. It is calculated by using the formula:

PLS = Germination% X Purity % / 100.

For imbibition, four replicates of 40 seeds to each were weight before and after imbibition. Seeds were imbibed in 100 ml of water for 72 hours and measurement were taken 3 times i.e., after every 24 hrs. Seedling length was taken after the completion of germination period (7 days) in randomly selected five seedlings from each replication. The dry weight of the 5 randomly selected seedlings (without cotyledons) for each replicate was measured after it was dried on oven at 103^{0}C for 24 hrs.

Results and Discussion

As quality is considered superior, if pure seed percentage is above 98, and other seeds and inert matter percentage as low as possible. Germination test are based on pure seed components, this has been shown by the observations recorded and that purity analysis and germination tests compliment each other. Thus the actual planting value of seed can be determined only when the purity analysis and germination tests are considered together. Vigour test have been used as complementary information to the germination test. They are considered efficient to classification of seed lots according to physiological potential, but it is also desirable that they provide coherent results with field seedling emergence.

Recorded experimental findings (Table 1) showed that sample S2 exhibited maximum germination, PLS and seedling length values. Maximum purity percent, highest imbibition and seedling emergence in minimum days was observed in Sample S4 and S1 obtained maximum (0.97 gm) dry weight of seedling. Seeds of sample S4 showed high emergence percent at the first reading with high rate of imbibition due to

the characteristic of high accessibility moisture seed coat. Similar responses have been described by McDonald & Copeland (2004). For early seedling emergence all samples were started taking observation from the 3rd day of sowing, on the 1st day of counting sample S4 and S3 recorded the maximum and minimum seedlings respectively. But there was no significant difference on the final counting date of seed germination except S3, having low vigour seed, which support the report of O'DELL *et al.* (1998). Sample S2 exhibited maximum germination, PLS and seedling length values with high seedling dry weight indicated more vigour than any other samples which was in accordance of the reports of Egli *et al.* (1990), Egli & TeKrony, (1996) and Marcos Filho (1999). The result of low emergence percentage contradicts the observation of Green *et al.* (1965), who reported that low emergence percentages in the laboratory and field were associated with high occurrence of green cotyledons in soyabean.

Table 1. Mean values of analysis by different tests methods of soyabean seed quality. In the table, seed lot 2 (S2) and lot 4 (S4) recorded maximum mean values in varied tests.

SS	P %	I (after 72 hrs)	G (%)	PLS %	E %	P. ht. (cm)	SDW (gm)
S1	94.52	6.83	95	89.79	99	16.60	0.97
S2	98.15	6.28	97	95.21	96	17.18	0.94
S3	97.63	6.80	82	80.06	95	10.43	0.57
S4	98.43	7.29	96	94.49	99	16.45	0.93

Acronym used: SS=Seed Sample, P=Purity, I=Imbibition, G=Germination, PLS=Pure live seed, E=Emergence, P. ht.=Plant height, SDW=Seedling dry weight

Thus, from the discussion it may be concluded that seed lot 2 (Sample S2) possessed the highest quality than any other seed lots but all the remaining three seed lots were found good enough to use as a planting material in the next season as the quality value was within the standard value of Indian Minimum Seed Standard.

Acknowledgement

The authors would like to thanks the progressive farmers for providing the seed materials and for their cooperation.

References

McDonald, M.B. & Copeland, L.O. Seed Science and Technology, Laboratory Manual. 1st Ed. 2004, Pub. Scientific Publishers (India).

Hartmann, H.T. and Kester Fred, D.E. Plant Propagation-Principles and Practices. Vol.1, 1999.

Egli, D.B. and D.M. Tekrony. Seedbed conditions and prediction of field emergence of soyabean seed. Journal of Production Agriculture, v.9, 1996, Pp.365-370.

Egli, D.B., TeKrony, D.M. and Wiralaga, R.A. Effect of soyabean seed vigor and size on seedling growth. J. Seed Tech. 1990, 14:1–12.

Green, D.E., Pinnell, L.E., Cavannah and Williams, L. F. Effect of planting date and maturity date on soyabean seed quality. *Agron. J.* 1965, 57:165-168.

Marcos Filho, J. Testes de vigor: Importância e utilização. In: Krzyzanowski, F.C.; Vieira, R.D.; França Neto, J.B. (Ed.) Vigor de sementes: conceitos e testes. Londrina: ABRATES, cap.1, 1999, Pp.1-21.

O'Dell, A., Scarisbrick, D. H. and Baker, D. A. Production of Belarussian soyabeans (*Glycine max*) in the UK: predicting time to emergence. Research Article: Crops and Soils, 1998.

3/27/2009

Conservation status of the endemic orchid *Peristylus kumaonensis* Renz. (Orchidaceae) of Western Himalaya, India

Jeewan Singh Jalal[*], Pankaj Kumar, [**] Lalit M. Tewari* and Y.P.S.Pangtey[*]

[*] Department of Botany, Kumaun University, Nainital, Uttarakhand
[**] Habitat Ecology Department, Wildlife Institute of India, Dehradun Uttarakhand (India)

Name of Author	E-mail address
Jeewan Singh Jalal	: jeewansinghjalal@rediffmail.com
Pankaj Kumar	: pankajsahani@rediffmail.com
Lalit M. Tewari	: l_tewari@rediffmail.com
Y.P.S.Pangtey	: y_pangtey@yahoo.com

ABSTRACT

Peristylus kumaonensis Renz is an endemic taxon of western Himalayas. It is found in the outer fringe of the Kumaun Himalayas 5 km away from Nainital towards Ratighat. The orchid species appears to be restricted to this area according to past and the present surveys. Major threats to the existence of this species are due to habitat fragmentation, forest fire and might be the invasion of a fern species *Phytopteris oxyloba*. This species is of conservation concern because of its low numbers of individuals and restricted distribution in the western Himalayas. [Nature and Science. 2009;7(5):86-89]. (ISSN: 1545-0740).

Keywords: Kumaun Himalayas, endemic, *P. kumaonensis* Renz, Western Himalayas.

INTRODUCTION

Peristylus is an Indo-Malaysian genus of about 60-70 species of terrestrial orchids. The genus is distributed in the tropical and sub-tropical parts of Asia, New Guinea, Australia and some Pacific Islands. The generic name is derived from Greek peri= around and stylus=column, referring to the shape of the column. The genus Peristylus having convex stigmas that are entirely united to the base of the labellum and to the auricles of the column. All species are multi-flowered with mostly dull coloured, small flowers held close to the flower stem which are short-lived. All the species are normally deciduous terrestrials with fleshy, subterranean tubers closely related to Habenaria. Peristylus is represented in India by 28 species with them many species are native to India. Eight species are found in western Himalayas. Kumaun Himalaya occupies in the central sector of Indian Himalaya and lies between 28°44'- 30° 49' N Lat. and 78° 45'- 81° and 01' E long. Broadly the area consists of three parallel mountain ranges. The outermost range rises steeply above the plains to more than 2000 m above msl, reaching 2600 m in some peaks near Nainital. Rainfall is heaviest on the southern slopes of this range, between 1981 cm and 3048 cm annually. This area receives the major part of its annual precipitation during the southwest monsoon from June to September. Altogether 192 species of orchids under 61 genera were recorded so far from Kumaun Himalaya (Pangtey et. al., 1991). ***Peristylus kumaonensis*** Renz was first time reported by Dr. J. Renz in 1983 from the locality 5 km from Nainital towards North on the way of Ratighat at altitude 2178 m and it is restricted to this area in the whole of western Himalayas (**Fig. 1 & 2**). That time almost 130 individuals were counted at this particular locality (Pangtey, personal communication). During our orchid study in Kumaun region since 2002 we are continuously observing the population of the species. Now the scenario of the whole area has been changed due to habitat changes and anthropogenic pressures. The population drastically changed only 30 individuals so far observed in this locality. The species is generally grows on the rocks covered by thick mosses bed. The mossy bed basically holds moisture and soil which is sufficient to the growth of the species. During our survey we tried to explore other area where the possibility of the occurrence of this species but we could not get this species in other part of Kumaun Himalayas. Thus it is very important to conserve the novel endemic orchid and its habitat.

SPECIES DESCRIPTION

Peristylus kumaonensis Renz., J. Orchid Soc. India 1: 23. fig. 1. A-H (1987); Pangtey et. al., Orchids Kumaun Him. 77 (1991); Jalal, Sys. Phyt. Hab. Eco. Orch. Utt.: 149 (2005).

Terrestrial herb with almost straight stem and usually 2-leaves, which are somewhat clasping and located near the base, leaves erect and unequal in size and shape. Lower leaves longer, narrowly oblong, acute, 9 x

1.5 cm. Upper leaves lanceolate to linear-lanceolate, reduced in size. Inflorescence very narrow, rather laxly sub-secund. Bracts lanceolate acuminate as long as the ovary or little shorter or longer. Flowers minute, greenish, and glabrous. Sepals converging. Dorsal sepal elliptic, obtuse, upto 1.7 mm long. Lateral sepals obliquely ovate-elliptical, acute, slightly longer than the dorsal. Petals obliquely rhomboid-elliptical, upto 1.5 mm long. Lip as long as the petals, trilobed near the middle, with small triangular side-lobes and a longer, obovate to oblong mid-lobe. Spur much shorter than the ovary (**Fig. 2**).

Flowering: This taxon flowers from late July through August.

Geographical Distribution: It is only known from on the way to Ratighat near Nainital, Western Himalaya.

Specimen Examined: Wildlife Institute of India herbarium (WII) - *J.S.Jalal 13993*.

Habitat: This taxon has been found at elevations of 2178 m (7145 ft) on the moist rocky surface covered by thick mosses patches. This particular habitat is a transition zone of Banj-oak (*Quercus leucotrichophora*) forest and Chir-pine (*Pinus roxburghii*) forest. Apart for this a fern species *Phytopteris oxyloba* is the main associate species.

THREATS

This species is threatened due to its low numbers of individuals and restricted distribution in Kumaun Himalayas. The plants are small and fragile and can not tolerate and direct impacts by anthropogenic pressures, canopy exposer and forest fire. Chir-pine forest is also rapidly encouraging the entire area, which is prone to fire. This forest also changes the hydrology of the area which impacts many rare herbs including orchids. The soil chemistry also gets changed under Chir-pine forest. The soil becomes more acidic under chir-pine forest which can put negative impact on the germination of orchid seeds. Another major threat is its road side location. This particular species is growing in the main Nainital-Ratighat road and which is frequently used by local community for their daily uses. During rainy season, that is also the peak flowering season of Peristylus ***kumaonensis*** Renz. The local villagers clean the road side tall herbs and bushes because of this activity most of the time the ***Peristylus kumaonensis*** Renz species also damaged. In past few years it was observed that *Phytopteris oxyloba*, a lithophytic fern also encroaching the habitat, resulting the whole rocks now covered fully with this fern. Most of the moist part of the rock encroached by this. For conservation of this endemic orchid further survey is required to relocate this species and establish its vulnerability to the threatening processes in the area. Recovery actions such as monitoring, fire management and habitat condition may need to be implemented and it is also recommended to include national threatened species list.

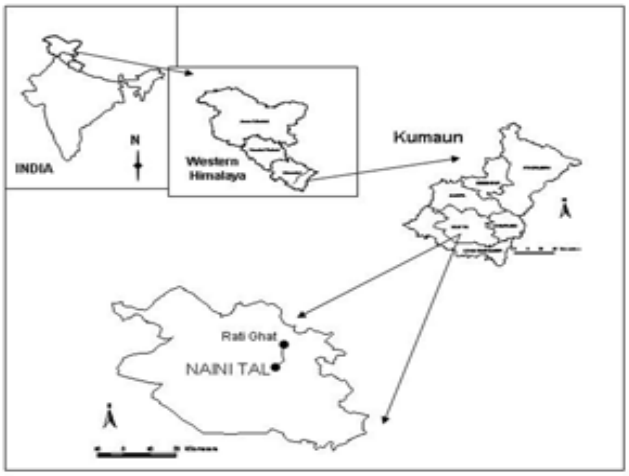


Figure 1: Showing location of *Peristylus kumaonensis* Renz **in Western Himalaya**

Figure 2: Habitat of *Peristylus kumaonensis* Renz.

Figure 3: *Peristylus kumaonensis* Renz: 1. Plant in habit; 2. Closeup of Inflorescence.

Figure 4: Close-up of Inflorescence

ACKNOWLEDGEMENTS
Authors are grateful to the Department Science and Technology, Government of India for financial support to carry out this work. Authors are also thankful to Director, Wildlife Institute of India for providing necessary support and facility.

Correspondence to:
Dr. Jeewan Singh Jalal
Young Scientist (DST)
Department of Botany, D.S.B.Campus
Kumaun University, Nainital,
Uttarakhand, India
Tel: 09412348q46
E-mail: jeewansinghjalal@rediffmail.com; jeewan.orchid@gmail.com

REFERENCES

Pangtey YPS, Samant, SS, GS Rawat. Orchids of Kumaun Himalaya.Bishen Singh Mahendra Pal Singh, Publication, Dehradun, India: 1991.

Jalal JS. Systematics, Phytogeography and Habitat Ecology of Orchids in Uttaranchal. Ph.D. thesis, Kumaun University, Nainital: 2005.

Renz J. *Peristylus kumaonensis* Renz., Journal of Orchid Society of India, 1987; 1 (23) fig. 1. A-H.

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