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VIBRATION MONITORING AND FAULT DIAGNOSIS OF AN I.D.FAN AT A CEMENT PLANT

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(Received 5 June 2002; accepted 12 September 2002)

I.D.fan (Kiln) of a Cement plant consisting of a motor, two mechanical couplings, a hydraulic coupling and a heavy fan (approx. two tons) was giving high vibrations at some locations. This was monitored at all the bearing locations and its vibration symptoms were recorded for analysis purpose. Frequency domain and phase analysis were made to diagnose the root cause of vibrations. On the basis of this analysis, fault observed was mainly with the fluid coupling. The vibration spectra and phase data are presented in this paper.

Key words: Vibration monitoring, Cement plant, Mechanical coupling, Rotating machinery.

Introduction

In the rotating machinery, vibrations have been adopted as a prime indicator of machine condition. During operation all machines are subjected to fatigue, wear and deformation which lead to the different faults like increase in the clearances between machine parts, shaft misalignment, cracks initiation and unbalances in rotors. All this leads to increase in vibration, which causes an additional dynamic load on bearings. The increasing vibration levels with time lead to the failure or breakdown of the machine. It is therefore, essential to get the vibration data and interpret it to determine the mechanical condition of a machine and pinpoint, any specific mechanical or operational defect (Ralph 1979; Edison 1994).

The vibration analysis of the I.D. fan (Kiln) of an Askri Cement plant at Nizampur NWFP is based on its time-to-time vibration monitoring. The vibration velocity is recorded along with phase in loaded condition at 1000-rpm fan speed. The collected data is analyzed by phase analysis technique. A data collector is used for frequency domain spectrum analysis.

Both the techniques are utilized to diagnose the problem. A brief introduction of the I.D.fan is given for understanding the system. Finally the problems in different machine components are diagnosed and discussed in detail.

Materials and Methods

The induced draught (I.D.) fan of the Cement plant is shown in Fig 1. The points 1,2,3,4,5 are the bearing locations, where the vibration data were collected in different directions, vertical, horizontal and axial with the help of a data collector. The whole system consists of a motor, two mechanical couplings a hydraulic coupling and a big fan.

*Author for correspondence

The I.D.fan drags high temperature air and dust to the Kiln with full load capacity of 375000 m^3/h at 350°C and 800mm of H_2O. It has 1200 kW motor driving the fan at the speed of 1000 rpm. The hydraulic coupling can control the fan speed from 200 to 1000 rpm.

The variable speed fluid coupling is hydraulic power transmission, which transmits power through fluid medium and releases stepless speed regulation. It consists of an impeller connecting with an input shaft, a turbine connecting with the output shaft and a rotating housing enclosing the turbine.

Vibration analysis. The system was monitored and diagnosed the fault on the basis of vibration spectra and phase analysis. It is discussed here.

Frequency spectrum analysis. The analysis of vibrations is said to be in the frequency domain and displays vibration amplitude along y-axis and frequency along x-axis in the spectrum. On the other hand, a plot of vibration amplitude against time, a waveform plot is said to be in the time domain. Energy in a vibration signal is distributed over a range of frequencies consisting of the fundamental frequency and complexities of the harmonics (Ralph 1979).

The presence of mechanical problems like misalignment of couplings, imbalance of shafts, worn damaged or locked coupling, bent shaft condition, bearing conditions and gear problems may be analyzed on the basis of vibration magnitude of different frequency peaks available in the frequency domain spectrum of the machine. The frequency spectrum may be analyzed on the following basis (Ralph 1979; Edison 1994; SKF 1995)

The presence of 2X peaks that is high in the radial direction is an indication of parallel misalignment. In almost 80% cases

of misalignment there exist 1X symptom. 2X appears in case of severe misalignment. The presence of high 1X and 2X peaks in the axial plane is the reason of angular misalignment.

A bent shaft problem is possible if vibration levels are relatively high in the axial plane across the component. The 1X peak will exist in the axial plane at two ends of a component.

If there exists a 1X peak in the axial plane and 1X,2X peak in the radial direction of a component, it will be an indication of worn, damaged or locked coupling.

The presence of high 1X frequency component in both the horizontal and vertical planes and readings differ from each other by a factor of 2. It is an indication of imbalance, possibly an eccentric rotor.

Mechanical looseness can take many forms in rotating machinery. The classic spectral signature is the string of harmonics of the running speed, with peaks ranging from 1 X to 10 X. These peaks can be accompanied by 1/2and 1/3 harmonics (i.e. peaks at 2. ½ X , 3 .1/3 X etc.). A looseness caused by structural defects (i.e.weakness in the machine feet, foundation or grouting) will more likely to have vibration signal with relatively higher peaks at 1X and 2X frequency condition. A looseness exhibited by relatively higher amplitudes in the upper harmonics (3X to 10X) is more likely to be the bearing related (given that machine has rolling element type bearings).

The presence of a peak in the approximate area of inner and outer race on a rolling element bearing of unknown configuration may indicate bearing wear. The non-synchronous peaks between 3X and 6X show the bearing problem.

The presence of sub-synchronous and low band symptoms on both ends of the shaft or machine component is a possible indication of structural resonance.

*Phase analysis.*The ability to determine the relative motion between various parts of a machine structure using phase measurements can be a very valuable analysis tool. For example high amplitudes of axial vibration may be caused by coupling misalignment, bearing misalignment and Bent shaft or unbalance of an over hung rotor (Ralph 1979).

The vibration signal at 1X and 2X can be obtained along with phase for detailed analysis.The first step is to select a convenient location on the machine where phase reading can be observed with the help of a photo pickup/strobe light. The objective of a phase analysis is to distinguish among imbalance, eccentricity, misalignment, bent shaft and mechanical looseness. The phase reference measurement accuracy lies within 30° (Bulk Kruger and SKF 1998). To analyze the imbalance, it is essential that 1X must be the dominant frequency of the spectrum. If this condition of frequency peaks along with the phase information is available, then it is better to segregate the problem by phase analysis. Following will be the conditions of phase relationship for evaluating a particular problem:

There will be a 90° phase shift, if the vibration sensor is moved from horizontal to vertical position on a machine. No radial phase shift may be observed across the machine or coupling. This will be the case of mass unbalance in the rotor.

If 180° phase shift is observed in the axial direction across the machine with no phase shift in the radial direction, this exhibits the bend shaft condition.

A phase shift of 180° in the axial direction will exist across the coupling in case of angular misalignment.

If 180° phase shift exists in radial direction across the coupling and 0° or 180° phase shift exists from horizontal to vertical position on the same bearing, this may be due to parallel misalignment.

The problem of both angular and parallel misalignment may appear, if phase shift acrosses the coupling in radial and axial direction which is about 180°.

Results and Discussion

The frequency spectrum analysis and diagnosis. The vibration velocity (mm/sec, peak value) was measured using a data collector (Microlog by SKF) with the help of a hand held accelerometer (4370 by B&K). The motor of the I.D.fan was monitored, while uncoupled. The levels of 1X rpm peaks were very much low.

The vibration measurements were made at point no.2, 3, 4, and 5 of the I.D. fan in vertical, horizontal and axial position, respectively in the loaded condition. The frequency spectrums obtained at all points were analyzed on the basis of theoretical background as discussed in article 3(a). The problems diagnosed are given below.

The high 1X peaks level along with 2X peaks in radial direction across the points 2 and 3 (Fig 2 and 3) is the indication of parallel misalignment in rigid coupling 1.

The high 1X peaks along with 2X peaks in the radial as well as in axial direction across the flexible coupling at point 3 and 4 (Fig 3 and 4) is an indication of misaligned, worn, ceased or locked coupling.

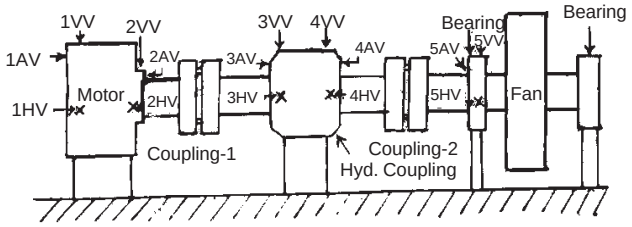

Fig 1. I.D.Fan (Kiln)

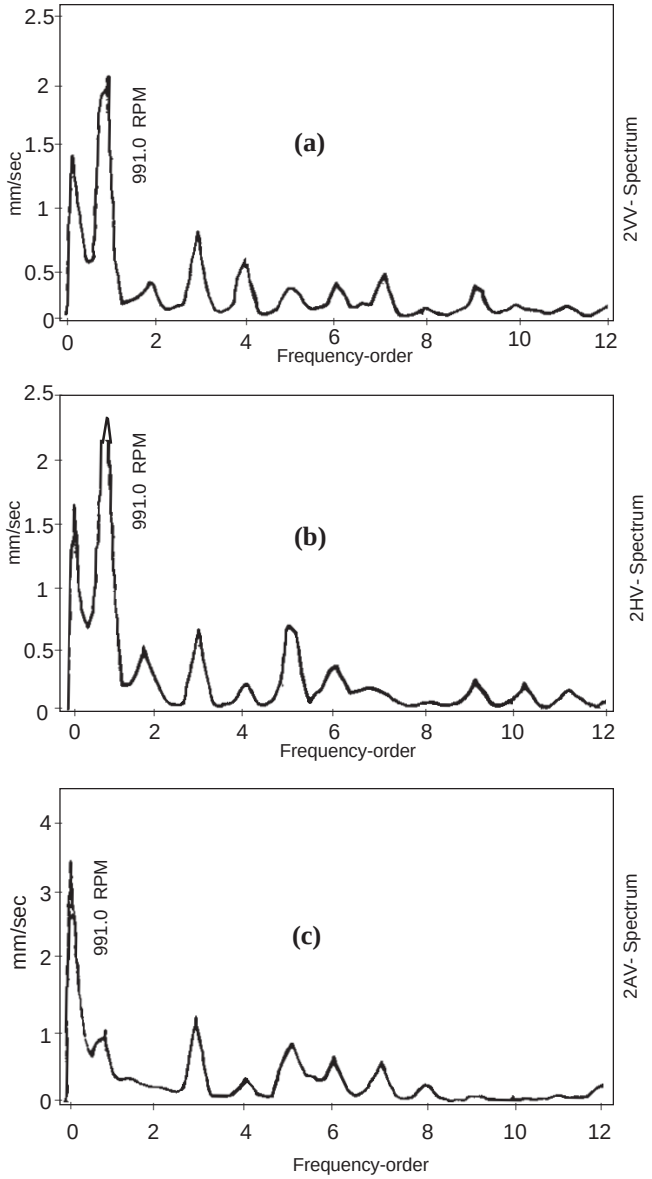

Fig 2 (a,b,c). Frequency domain spectrums at point 2 of I.D. fan.

The high 1X peaks along with 2X peaks in the radial as well as in axial direction across the points 4 and 5 (Fig 4 and 5) is indication of both parallel & angular misalignment in rigid coupling-2

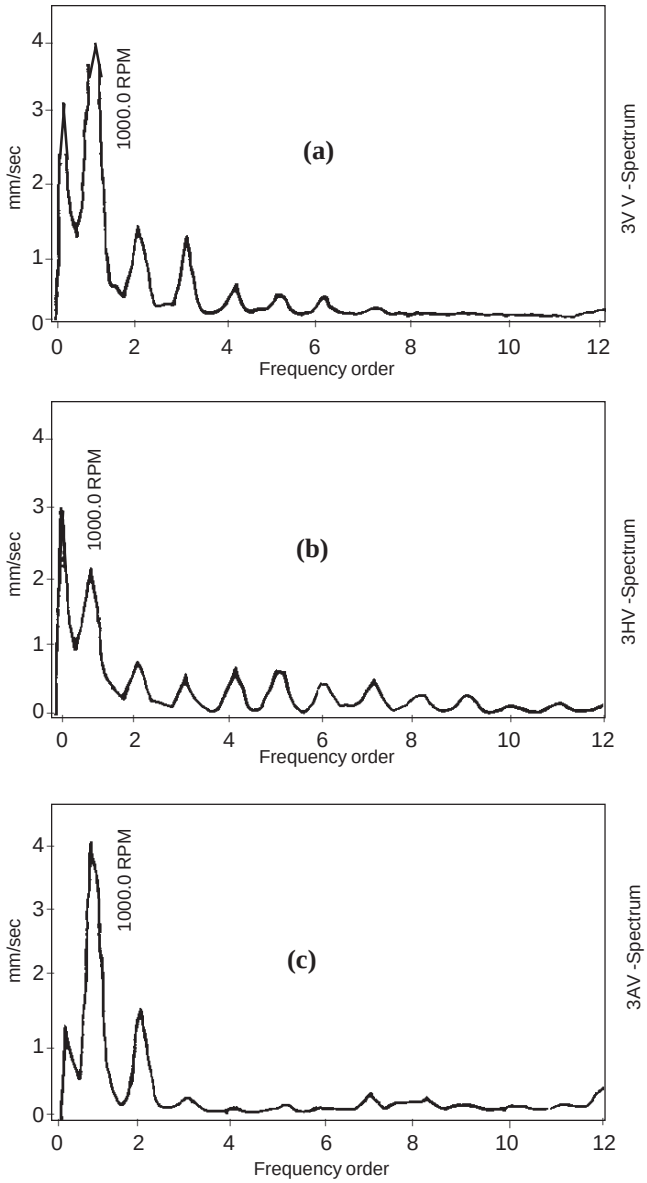

Fig 3 (a,b,c). Frequency domain spectrums at point 3 of I.D.fan.

The string of peaks at 1X, 1.5X, 2X, 2.5X and so on, almost in all points spectrum is the clear indication of mechanical looseness in all machine components or its foundations.

The subsynchronous peak observed in all spectrums is the indication of structural resonance. This may be the frequency of any part in the machine system or may be due to vibration in the nearby machine. These low frequency peak remain even if the system is made OFF. This rules out the possibility of outer race problem in the bearings

The high 1X peaks in both radial and axial planes at about all points may also be due to imbalance and bent shaft condition in the three coupling.

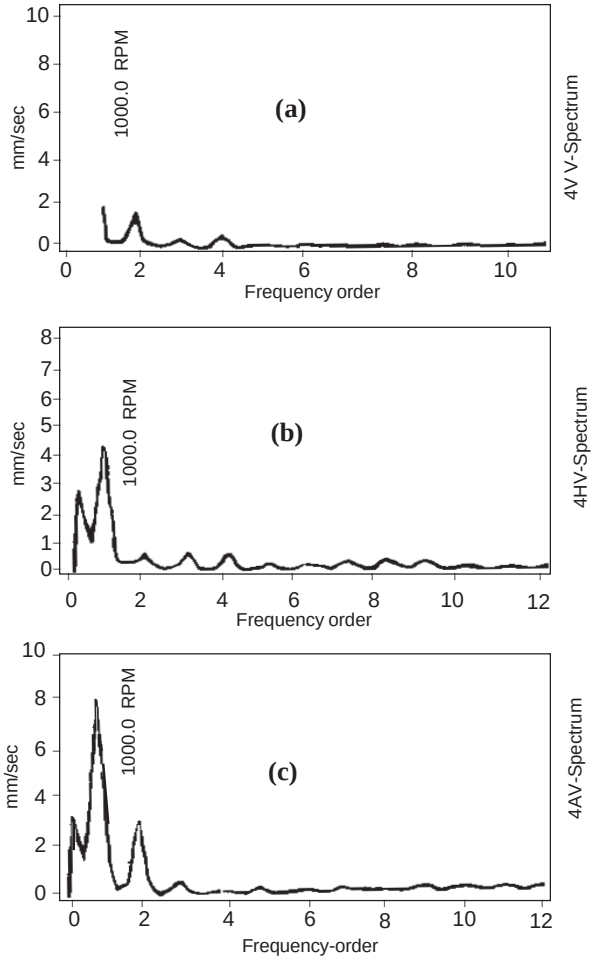

Fig 4 (a,b,c). Frequency domain spectrums at point 4 of I.D.fan.

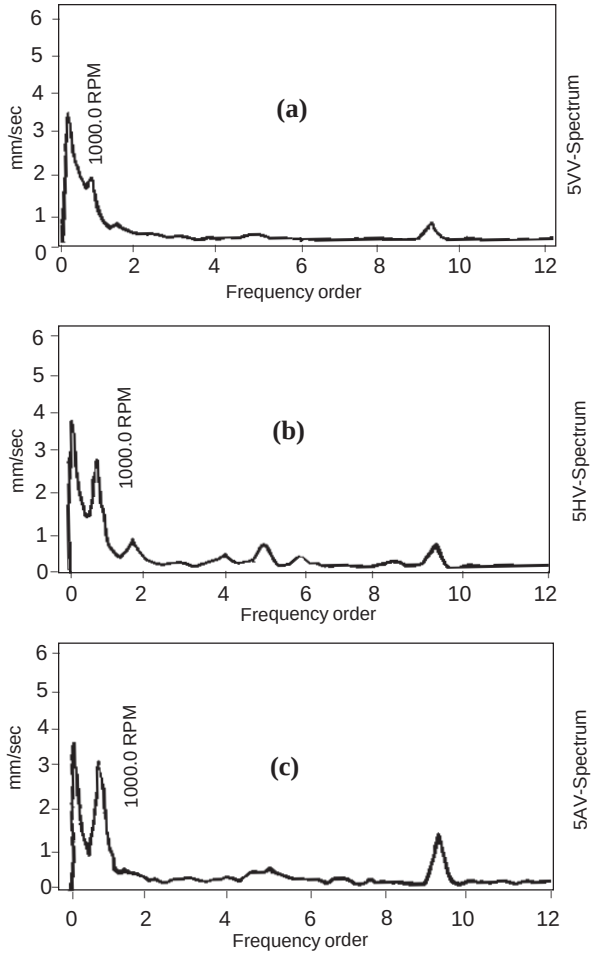

Fig 5 (a,b,c). Frequency domain spectrums at point 5 of I.D.fan.

Table I
The measurements of the vibration severity levels

S.No.	Pickup location	SUM mm/sec	1X RPM mm/sec with phase
	V	1.20	0.28/207°
1	H	1.70	1.00/216°
	A	3.70	0.15/330°
	V	2.00	1.50/207°
2	H	2.50	2.00/102°
	A	3.00	0.50/12°
	V	4.10	2.00/160°
3	H	5.10	2.20/299°
	A	9.80	4.10/200°
	V	6.00	1.30/146°
4	H	5.20	1.60/325°
	A	8.90	4.30/358°
	V	2.30	0.40/304°
5	H	2.50	0.10/394°
	A	4.40	0.90/325°
	V	0.95	0.78/129°
6	H	1.50	0.67/328°
	A	2.10	1.70/315°

*The phase analysis and diagnosis.*The vibration data of the I.D. fan was collected by another vibroport(Vibrometer by Schenck) with the help of a hand held velocity sensor by measuring vibration severity level (velocity in mm/sec, rms value) at all the located points in vertical, horizontal and axial direction as shown in Fig 1.The vibration data for overall and 1X frequency component along with phase were recorded for analysis purpose.

The vibration severity levels measured (Table 1) at all machine components of I.D.fan, except the hydraulic coupling, which is of low level and does not generate the symptom of any severe problem. The vibration signal across the two ends of hydraulic coupling and rigid coupling-I may be analyzed as under.

The 1X peak of moderate level along with the phase shift of 197° in horizontal direction across the rigid coupling-1 at point 2 and 3 is the indication of parallel misalignment in this coupling. This can be the problem of worn coupling which can be confirmed after its dis-assembling.

It may be noted that vibration levels obtained during phase measurement do not meet with the values of spectra. This is due to the use of another instrument on different date for phase analysis.

The high axial and moderate horizontal 1X peak at point 3 and 4 across the fluid coupling along with the phase shift of about 180˚is an indication of parallel as well as angular misalignment in this coupling

Conclusion

On the basis of frequency spectrum and phase analysis, it is diagnosed that:

There is maximum chance that problems of misalignment or wear exist in the hydraulic coupling.

There is an indication of moderate misalignment in both the rigid couplings.

The 1X peak along with higher harmonics at about all machine components is due to mechanical looseness in most of the machine components or its foundation.

The subsynchronous component present in almost all spectrums is because of structural resonance. This may be due to vibration in the nearby system or the natural frequency of a part of any machine in the system.

Any loose mechanical component or foundation is to be checked and rectified. The rigid and hydraulic couplings require adequate rectification. The machine foundation and vibration isolators are also to be checked.

The vibration data of I.D.fan system may be taken again as the rectification work is completed.

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SPECTROPHOTOMETRIC METHOD OF THE DETERMINATION OF GOLD (III) BY USING IMIPRAMINE HYDROCHLORIDE AND PROMETHAZINE HYDROCHLORIDE

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Imipramine hydrochloride [IPM·HCl] and promethazine hydrochloride [PMT·HCl] were used for the spectrophotometric determination of gold (III) in the aqueous solution. The halides complexes of gold (III) created a coloured coupling with the studied drugs which were extractable in chloroform. These new compounds were characterised by IR, UV-VIS spectra and thermal and elemental analysis. Rapid and sensitive spectrophotometric method for the determination of gold (III) in the aqueous solution is described. The absorbance was found to be linear function of the gold (III) concentration in the range from 0.2 to 20 x 10^{-1} mg. The ratio of complex $[AuX_4]^-$ to the organic cation from drug in the obtained compounds was determined as 1:1. The method was satisfactorily applied to the analysis of gold (III). A great advantage of the proposed method is that the trace amounts of gold (III) can also be examined.

Key words: Imipramine hydrochloride, Promethazine hydrochloride, Gold(III) halides complexes.

Introduction

One of the aims of contemporary analytical chemistry is the elaboration of more rapid, sensitive and efficient methods of determination of compounds and elements. It is known, that the derivatives of the 5H-dibenz[b,f]azepine and phenotiazine are created with some metals complexes, the coloured coupling (Dembinksi 1977; Tarasiewicz *et al* 1999; Misiuk 1999; Ramappa 1999). There are imipramine hydrochloride and promethazine hydrochloride in these groups. These compounds are used as the psychotropic drug in the medicine (Pawelczyk 1986). However, they were used most often for the determination of trace amount of some metals (Dembinski 1983; Puzanowska-Tarasiewicz 1998; Dembinski and Szydlowska-Czerniak 1999).

Many analytical procedures are used for determination of Gold (III). They are gravimetric (Donova and Siftar 1993), titrimetric (Abramovic B.F. *et al* 1999), voltammetric (Basu *et al* 1980) and spectrophotometric methods (Prakash *et al* 1986; Rakhmatuullaeu and Giyaru 1989; Patroescu *et al* 1989; Gurjeva and Savvin 1989). Moreover, the new methods of gold (III) determination are used, often. However these methods, such as electrochemical (Karolczyk 1996), AAS (Zaikm *et al* 1989) and neutron scattering (Ivanenko *et al* 1989), are expensive.

The aim of the present paper is to elaborate quick and well method of determination of gold (III) in the aqueous solutions. Au (III) forms negatively charged anionic complexes with the halides (Cl, Br, I). Anionic gold(III) halide complexes with promethazine [10-(2-dimethylaminopropyl) - phenothiazine] and imipramine [{5-(3-dimethylaminopropyl)-10, 11-dihydro-5H-dibenz [b, f] azepine}] in the hydrochlorides form can be used for a new complexing agents (Gowda and Ramappa 1976). The obtained compounds were intensively coloured and thermal stable, so they can be applied in a spectrophotometric and weight methods of gold(III) determination. The obtained coupling will be characterised by IR and UV-VIS spectra and elemental and thermal analyses.

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Experimental

Reagents: Imipramine hydrochloride (99%) -aqueous solution: 0.1 x 10^{-1} mol and 1.0 x 10^{-4} mol, Promethazine hydrochloride (99%) aqueous solutions: 0.1 x 10^{-1} mol and 1.0 x 10^{-4} mol, Promethazine hydrochloride (99%) and imipramine hydrochloride (99%). These were purchased from Aldrich and used without purification.

The concentration of the gold as $[AuCl_4]^-$ was 3.2931 x 10^{-2} mol.

Gold atomic absorption standard solution: 1 µg of Au in 5 wt.% HCl, Potassium iodide - solution 10%, Potassium bromide - solution 10%. All used chemicals were analytical grade purchased from POCH Gliwice.

Apparatus: Spectrophotometer UV-VIS-Shimadzu 1601 PC; Spectrophotometer IR; -Spectrum 2000 FT Perkin Elmer; Derivatograph - MOM-OD-102; Spectrometer AAS Philips PV 9100X.

Preparation and composition of the halide complex of gold (III) with the drugs: The 100 ml of the 3.293 x10^{-2} mol x 1^{-1} tetrachloroaurate (tetrabromoaurate, tetraiodoaurate)

were added to the 10 ml of 2 x 10^{-1} mol of sulphuric acid and 100 mol of 0.1 x 1^{-1} mol imipramine hydrochloride (or promethazine hydrochloride). The drugs were added by small portions the reaction solutions were intensively mixed, the precipitates were formed very quickly. The colours of these compounds changed from the dark brown to the light brown, The obtained precipitates were filtered, washed with distilled water and recrystallized from methanol. The pured precipitates were studied by the elemental analysis and the IR spectroscopy.

Results and Discussion

Elemental analysis: The results of the elemental analysis are presented in the Table 1. Element analysis established the composition of the obtained compounds. The ratio of the organic cation from the drug to the gold (III) complex in the obtained precipitates was 1:1. On the basis of elemental analysis, obtained coloured compounds have the formulas [IPM H]·[AuX_4] and [PMT H]·[AuX_4], where X = Cl, Br, I.

The IR spectra. Infrared spectra were analysed to establish the character of interaction between components of the obtained species (Wojtkowiak and Chabanel 1984). These spectra were obtained using the pressed pellets of the investigated compounds with potassium bromide (ratio 3/300) and as the thin films on the polyethylene plate. The clear differences are in the range of far IR. Figure 1 presents spectra of the promethazine hydrochloride, imipramine hydrochloride and their couplings with the tetrabromoaurate complex, for example. As results from these spectra the characteristic bands of the drugs have the different positions and intensity. However, in the case of their couplings with the tetrabromoaurate ion, the spectra are the same practically. This in fact indicates, that the strong bands in this range comes from [$AuBr_4$]$^-$ (Puddephatt 1978).

The differences of the IR spectra in the higher range for the drugs and the studied coupling pointed on formation the new systems. Figure 2 presents IR spectra for imipramine hydrochloride and imipramine tetrabromoaurate. The characteristic bands for third order amine C_3N (weak in the range 2200-2500 cm^{-1}), are difficult to identify, but their hydrochlorides have the strong bands at 2500-2600 cm^{-1}. Others characteristic bands are marked by different bands. The bands characteristic for the drug hydrochloride are shifted towards lower energetic region in IR spectra of the coupling [IPM][$AuBr_4$] and their intensity which is lower. This effect is connected with the appearance of the strong interactions: Au-Br.....H-N-CH_2. Hydrogen bond which occurs in the crystal lattice reduces the N-H bond strength causing the above mentioned band shift. The spectroscopic investigations indicate that formation of these new compounds is the results of the exchange of chloride ion in drug for the greater gold (III) halide complex.

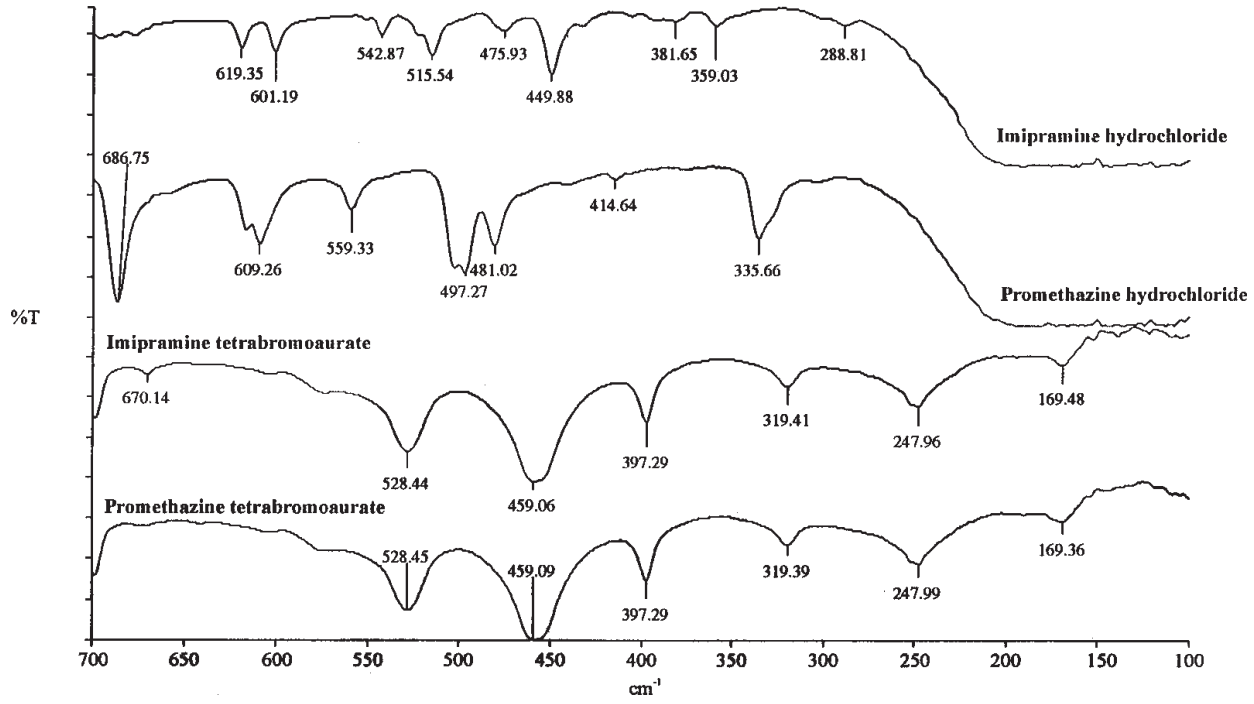

Fig. 1 IR spectra for imipramine hydrochloride, imipramine tetrabromoaurate, promethazine hydrochloride and promethazine tetrabromoaurate (thin film on PE).

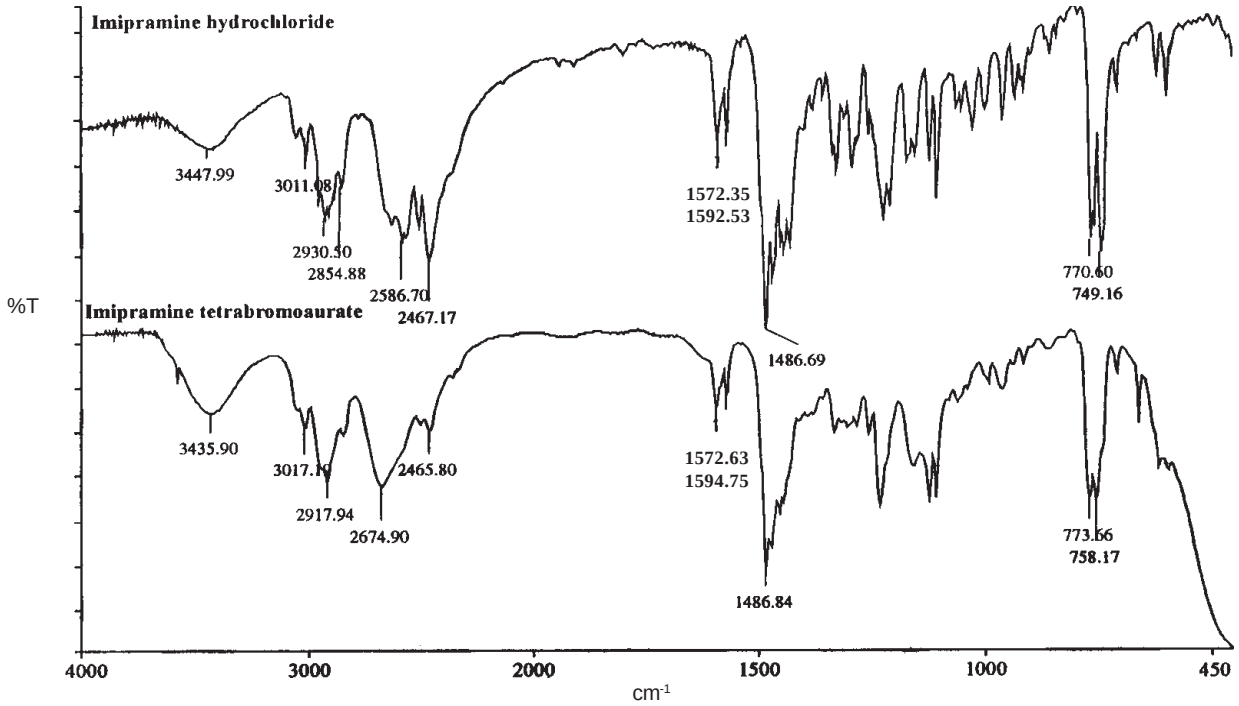

Fig. 2 IR spectra for imipramine hydrochloride and imipramine tetrabromoaurate.

Thermal analysis: For the purpose to establish the thermal stability of the obtained couplings they were made thermal studies. The conditions of these studies are as follow: the nitrogen atmosphere with the heating rate 2.5˚C x min^{-1}, the temperature range from 20 to 500 ˚C, the weight of the samples -5 mg. The sensitivity of DTA and DTG was 1/3. In Figure 3, for example, the thermal curves of [PMT·H] · [$AuBr_4$] are presented.

All the curves have the similar runs. The runs of TG and DTG point that created couplings are about to stable [PMT.H]. [$AuCl_4$] -230 ; [PMT·H] · [$AuBr_4$) - 220 ˚C; [PMT·H] · [Aul_4]- 200 ˚C; [IPM·H] · [$AuCl_4$] -215 ˚C; [IPM·H]·[$AuBr_4$] - 205 ˚C; [IPM·H] · [Aul_4] - 190 ˚C. The thermal degradation takes place at the different temperature depends on the gold (III) complex and the kind of drug.

The DTA curves of the studied compounds are more complicated. The large exothermic peaks which are before the melting and degradation temperature probably point all the intramolecular processes. The peaks at 215˚C for presented coupling point the melting point, what confirms the study on the Betius table.

Analysis of UV-VIS spectra: For the purpose of the useful application of the obtained compounds to the spectrophotometric method for determination of the gold (III), it was made the spectra in the range of UV-VIS. The spectroscopic studies were made for the coloured coupling of psychotropic drug with the chloride complexes of gold (III) as the solutions in chloroform. In Figure 4, for example, the spectra of imipramine hydrochloride and tetrabromoaurate complexes with imipramine, are presented.

Table 1
The results of the elemental analysis

Compounds	C %		H %		N %		S %	
	calc.	det.	calc.	det.	calc.	det.	calc.	det.
[PMT·H] [$AuCl_4$]	32.70	32.85	3.37	3.42	4.49	4.51	5. 13	5. 11
[PMT·H] [$AuBr_4$]	25.45	25.50	2.62	2.68	3.49	3.60	3. 99	4. 08
[PMT·H] [Aul_4]	20.61	21.01	2.12	2.10	2.83	2.90	3. 23	3. 20
[PMT·H] [$AuCl_4$]	36.79	36.82	4.03	4.05	4.52	4.48	-	-
[PMT·H] [$AuBr_4$]	28.59	28.65	3.13	3.15	3.51	3.54	-	-
[PMT·H] [Aul_4]	23.13	23.93	2.54	2.61	2.84	2.86	-	-

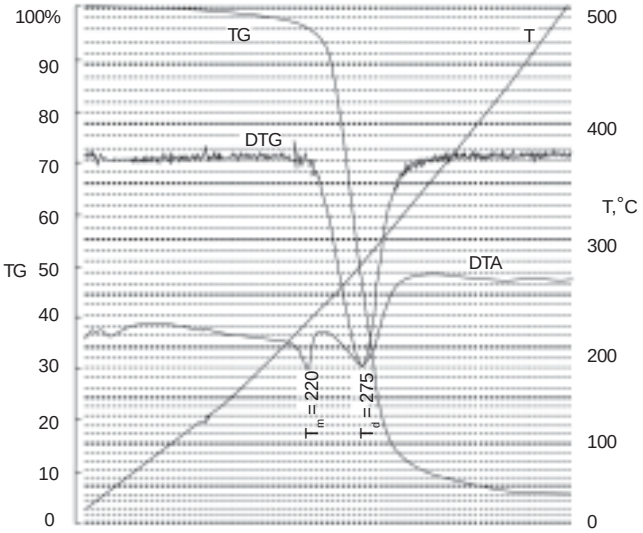

Fig. 3 The thermal curves of the imipramine tetrabromoaurate.

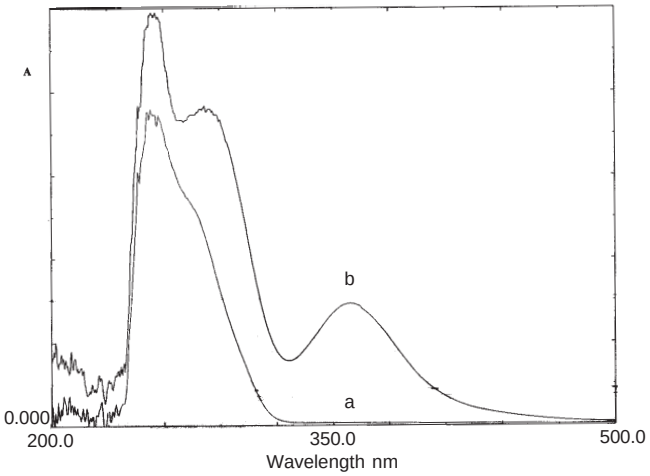

Fig. 4 UV-VIS spectra of imipramine hydrochloride (a) and imipramine tetrabromoaurate (b) (solution in $CHCl_3$).

The spectra in VIS region shows only one absorption band for all investigated compounds. This band is connected probably with the ligand - metal charge transfer (Lever 1984).

Determination of calibration curve: The solutions for the determination of the calibration curves were made for the gold (III) concentration from 0.02 to 20 x 10^{-1} mg. The gold (III) was in the form of tetrachloroaurate ion. Into the measuring flask were added 0.05 - 0.5 ml of 3.2931 x 10^{-2} mol; 0.05 - 0.5 ml of 3.2931 x 10^{-4} mol and 0.05 ml of 3.2931 x 10^{-6} mol; solution of tetrachloroaurate and 2 ml of the sulphuric acid (the concentration 2 x 10^{-1} mol). Next, it was introduced 2 ml of the drug solution (the concentration 1 x 10^{-4} mol). The contents were refilled with water to 10 ml and then was extracted with few portions of chloroform. In three times repeated extraction (each 2 extracts were filled up to 10 ml with chloroform. The chloroform solutions of studied couplings were stable for 48 hours.

The absorbance was measured at 517 nm for the promethazine coupling and at 667 nm for imipramine and was found to be a linear function of gold (III) chloride complexes in the range of 0.02 to 20 x 10^{-1} mg for promethazine hydrochloride and in the range 0.2 to 20 x 10^{-1} mg for imipramine hydrochloride. The molar absorptivity is found as $\varepsilon = 3.66 \times 10^4$ 1 x mol^{-1} for [PMT·H]·[$AuCl_4$] and $\varepsilon = 1.20 \times 10^3$ 1 x mol^{-1} x cm^{-1} for [IPM·H] [$AuCl_4$]. The data were elaborated using a linear least squares method (y = 36613.41x - 0.01247, correlation coefficient = 0.9947 for promethazine and y = 1197.35x - 0.02057, correlation coefficient = 0.9922 for imipramine).

Effect of foreign ions: Selectivity of the prepared spectrophotometric method for the determination of gold (III) was checked with the foreign ions. Results of these measurements indicate that the positive interference is caused by following ions: Cu(II), Pt(II). It was shown that the fivefold excess of the above ions influence on determination results and gives positive error 5% and more. Even fivefold and tenfold excess of such cations as: Ni(II), Co(II), Mg(II), Ca(II) and anions as: Cl^-, NO_3^-, SO_4^{2-} not interfere.

Comparison elaborated method with the AAS results: The elaborated method was tested on the gold atomic absorption standard solution: 1 µg of Au in 5% wt. HCl compared with the AAS method. The AAS method of gold (III) determi-

Table 2
Comparison determination of gold (III) with using promethazine hydrochloride and AAS method

Cotents of gold (III) added mg	found* mg	Standard deviation mgx10^{-4}	Relative standard deviation%	Confidence limits** mg
	a) 0.0178	5.27	2.95	0.0178 ± 6.54 x10^{-4}
0.02	b) 0.0201	1.58	0.78	0.0201 ± 1.96 x 10^{-4}
	c) 0.0199	1.58	0.79	0.0199 ± 1.96 x 10^{-4}
	a) 2.0009	1.55	0.01	2.0012 ± 1.92 x 10^{-4}
2	b) 2.0005	0.95	0.01	2.0002 ± 1.18 x 10^{-4}
	c) 2.0007	1.01	0.01	2.0007 ± 1.25 x 10^{-4}
20	a) 20.0049	1.83	<0.01	20.0049 ± 0.02 x 10^{-4}
	b) 20.0017	1.27	<0.01	20.0017 ± 1.58 x 10^{-4}
	c) 20.0015	1.34	<0.01	20.0015 ± 1.66 x 10^{-4}

a) determined with IPM* HCl, b) determined with PMT* HCl, c) determined by AAS, *for five determination, **probability level 0.95

nation was made to use the cavity cathode at 242.8 nm. These results are listed in Table 2.

As shown in Table 2 the elaborated method is comparable with the AAS method in the range 0.02 to 200 x 10^{-1} mg gold concentration. The results obtained for promethazine hydrochloride were better than for impipramine hydrochloride and in excellent agreement obtained from AAS.

Conclusion. It was shown that promethazine hydrochloride and impiramine hydrochloride react with halide complexes of gold (III) in acid medium, forming intensively coloured compounds. The results of thermal studies of obtained compounds point on their thermal stability.

It has been proved that these compounds are ionic pair salt of protonated promethazine and imipramine with the $[AuX_4]^-$ in the ratio 1:1. This fact confirms the results of the elemental analysis. Moreover, the IR spectra indicates that the coupling of drugs hydrochlorides with the gold (III) halogens complexes were formed by an exchange of chloride ion in PMT·HCl or IPM·HCl for $[AuX_4]^-$ ion.

The obtained compounds can be quantitatively extracted with chloroform. Taking into account a large stability of the complex in chloroform extract. The linear dependence was found for the concentration gold (III) from 0.2 to 20 x 10^{-1} mg. The studies show that it is possible to determine imipramine and promethazine hydrochloride using halogens complexes of gold (III). The presented method of the spectrophotometric determination of gold (III) is a quick and in good agreement with the AAS method.

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A FLAVONE FROM THE SEEDS OF *CARUM CARVI* L. (UMBELLIFERAE)

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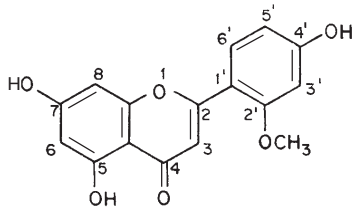
Chalcones constitute an important group of natural products and some of them possess a wide range of biological activities such as antibacterial, anti-fungal, anti-inflammatory, antimicrobial, anti-cancer, anti-tumour, postaglandin binding and insect anti-feedant (Hossain 2001). *Carum carvi* L. is reported for its medicinal values (Kirtikar and Basu 1995). In this paper, we describe the isolation of a flavone from the methanolic extract of the seeds of *Carum carvi* L. and its characterization as 4′, 5, 7-trihydroxy-2′-methoxyflavone (structure as given below).



The uncorrected melting point was determined using an electrothermal melting point apparatus (Gallenkamp). IR spectra were recorded (KBr discs) on a Pye- Union SP3-300 IR spectrophotometer (max: cm^{-1}), ^{1}H-NMR spectra on a Perkin Elmer R-32 (90 MHz) instrument in $CDCl_3$ with TMS as an internal standard (chemical shift in ppm). TLC was performed using silica gel 60G. Satisfactory elemental analysis was obtained for the compound and structures are in accord with the UV, IR and ^{1}H-NMR. Mass spectra were recorded on VG 7070 E analytical mass spectrometer.

The seeds of the spice *Carum carvi* L. (Bengali name-Jira; English name-Caraway) were collected from traditional spice seller of Ananda Bazar of Dhaka, Bangladesh in March 1997.

The seeds were cleaned by removing dust materials, then they were dried in sunlight for two days. Finally seeds were ground to powder form using the grinding machine. The dried and powdered seeds were extracted successively with n-hexane and methanol. Examination of the n-hexane extract revealed the pressure of waxy materials only. The methanolic extract (5.6 g) was fractionated by column chromatography over silica gel (60-120 mesh) using n-hexane-acetone-petroleum spirit (20:2:3) and acetone-n-hexane (5:27) gave the compounds 4′, 5, 7-trihydroxy-2′-methoxyflavone (0.175 g) and myricetine (0.093 g) (Islam and Hossain 1993).

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Compound (**1**) obtained from column was further purified by preparative TLC over silica gel 60G using n-hexane-acetone-petroleum spirit (12:5:2) as developing solvent. It was crystallized from petroleum spirit as yellow needles, m.p. 232°C (according to Islam and Hossain 1993, m.p. 230-234°C). Its identity as myricetine was confirmed by comparison (Co-TLC and m.m.p.) with an authentic sample.

Compound (**2**) obtained from column was further purified by preparative TLC over silica gel 60G n-hexane-acetone-petroleum spirit (19:2:1) as developing solvent. It was crystallized from petroleum spirit as yellow needles, m.p. 105°C. (M^+, 300m/z), Rf 0.67 (n-hexane-acetone-petroleum spirit 19:2:1); UV: 228, 245, 355 nm; IR: 3520, 2912, 2295, 1645, 1605, 1595, 1473, 1365, 1115, 1060, 980, 710 cm^{-1}; ^{1}H-NMR (DMSO-d_6,δ): 3.99 (s, 3H, -OCH_3), 6.31 (s, 1H, H-3), 6.45 (s, 1H, H-8), 6.98 (s, 1H, H-6), 7.12 (m, 3H, H-3', 5' and 6'), 12.75 (s, 3H, -OH). [Found: C, 64.00; H, 4.00. $C_{16}H_{12}O_6$ requires: C, 64.37; H, 4.30%]. It was identified as 4′,5,7-trihydroxy-2′-methoxyflavone.

A methanolic extract of the seeds of the spice *Carum carvi* L. yielded one known compound, myricetine and another unknown compound which is identified as 4′,5, 7-trihydroxy-2′-methoxyflavone. The known compound myricetine was identified by comparison of their spectral properties with those reported by Islam and Hossain (1993).

The new flavone analysed by VG 7070 E analytical mass spectrum for $[M]^+$ ion at 300m/z indicated an empirical formula of $C_{16}H_{12}O_6$. The characteristic infrared spectrum of 4′,5,7-trihydroxy-2′-methoxyflavone showed absorption frequencies at 3520 and 1645 cm^{-1}, indicating the presence of hydroxyl group and the ketonic group in conjugation and the absorption peaks at 1605 and 1595 cm^{-1} indicated the presence of unsymmetric ethylene double bond and the aromatic rings, respectively. The ^{1}H-NMR spectrum of the flavone indicated the presence of *O*-methyl unit. A sharp singlet at δ 3.99 revealed the presence of methoxy group at aromatic ring. A singlet at δ 6.31 indicated the presence of H-3 for the flavone nucleus. Two singlets at δ 6.45 and δ 6.98 indicated the presence of H-6 and H-8 and a broad singlet at δ 12.75 indicated the presence of three -OH groups, respectively.

Key words: *Carum carvi* L., Flavones, 4′, 5, 7-trihydroxy-2′-methoxyflavone.

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Short Communication

Pak. J. Sci. Ind. Res. 2003 ***46*** *(4) 236-238*

CONDENSATION OF SOME HETARYL SUBSTITUTED DIKETONES WITH AROMATIC DIAMINE

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The condensation of 1,2-di(quinolyl-2)-1,2 ethanedione and 1,2-di(6-methylquinolyl-2)-1,2-ethanedione with 4-methyl 1,2-phenylenediamine in the presence of acetic acid results in the formation of 2,3-di(quinolyl-2)-6-methylqui-noxaline and 2,3-di(6-methylquinolyl-2)-6-methylquinoxaline, respectively.

The biological activities of the nitrogen containing conjugated heterocyclic compounds have been found to draw the attentions of the researchers of modern times. Such compounds are considerably used pharmaceutically as antiulcer, antimalarial, tubercluocidal and sedatives besides their uses as dyes in the textile industry, pesticides, stablizers and inhibitors (Cheeseman & Werstuick 1978 and Cheeseman 1963).

Quinoxalines are the heterocyclic compounds that differ by the presence of a tertiary nitrogen atom in place of carbon at the fourth position in the heterocyclic ring of quinoline, are normally synthesized by the condensation of an aromatic o-diamine and ∝-dicarbonyl compound. These compounds are considered to have significant biological activitity. Their reactions as well as their pharmacological actions, continue to stimulate many investigations for example 2-methyl-quinoxaline N,N-dioxides substituted in the 3-position (e.g., with amide, amidino and ester groups) are potent bacteriocides (Ley, Eholzer, Metzger and Fritsche 1969, and 1970; Kasubick and Robertson 1972). Antibiotics of the triostin and quinomycin series, isolated from the cultures of *Streptomyces aureus*, have been shown by degradative study to contain a quinoxaline-2-carboxylic acid residue (Cheeseman & Werstiuck 1978). Keeping this in view, the present research was conducted to add some other compounds to this class.

2,3-Di(quinolyl-2)-6-methyl quinoxaline **(1a)** and 2,3-di (6-methylquinolyl-2)-6-methyl quinoxaline **(1b)** were synthesized by condensation followed by ring closure reaction, when 1,2-di(quinolyl-2)-1,2-ethanedione and/or 1,2-di (6-methylquinolyl-2)-1,2-ethanedione was treated with methyl substituted o-phenylenediamine respectively. The structures of the newly synthesized compounds have been determined and characterized by ultraviolet, infrared, nuclear magnetic resonance and mass spectral data whereas confirmed by elemental analysis.

Melting points were measured in open capillaries with an Electrothermal IA 9100 digital melting point apparatus.

All infrared spectra were recorded on a Phillips PU9714 Spectrophotometer using infrared grade potassium bromide. Nuclear magnetic resonance (^{1}H & ^{13}C) spectra were determined on "Varian 200MHz Gemini", "Bruker AC-200MHz FT-NMR" and "Bruker AM-500MHz FT-NMR spectrometers in deuteriochloroform and are reported in parts per million downfield from tetramethyl silane (TMS) as the internal standard (δ scale). Mass spectra were obtained with E1 MAT 312, Varian MAT 111, Varian MAT 112 and Hewlett Packard GC/MS 5890 spectrometer. Chemical analysis were performed in Austria and Germany and all new compounds gave satisfactory elemental analysis.

For the purpose of column chromatography silica gel 60 (70-230 mesh) from D. Merck AG was used. Eastman Kodak chromogram 13181 silica gel sheets with fluorescent indicator was used for thin layer chromatography (TLC).

The required 1,2-di(quinolyl-2)-1,2-ethanedione and 1,2-di (6-methylquinolyl-2)-1,2-ethanedione were prepared by the oxidation of 1,2-di (quinolyl-2)-1,2-ethenediol and 1,2-di (6-methylquinolyl-2)-1,2-ethenediol respectively, which were synthesized by the condensation reactions of the quinoline-2-carboxaldehyde and 6-methylquinoline-2-carboxaldehyde respectively. Quinoline-2-carboxaldehyde and 6-methylquinoline-2-carboxaldehyde in turn were prepared according to the literature procedure by the reaction of SeO_2 with the starting material i.e. 2-methylquinoline and 2,6-dimethylquinoline (Kaplan 1941) respectively. The obtained heterocyclic carboxaldehydes were found to have properties similar to that given in the literature. SeO_2 for the reaction was freshly prepared just before use by the method given by researchers (Blatt 1966).

All crude reaction products were examined by thin layer chromatography with chloroform as developing solvent and compared with the starting material and reagents to follow the progress of the reactions.

The ir, nmr, ms and analytical data alongwith the purification procedures are given in each experiment separately.

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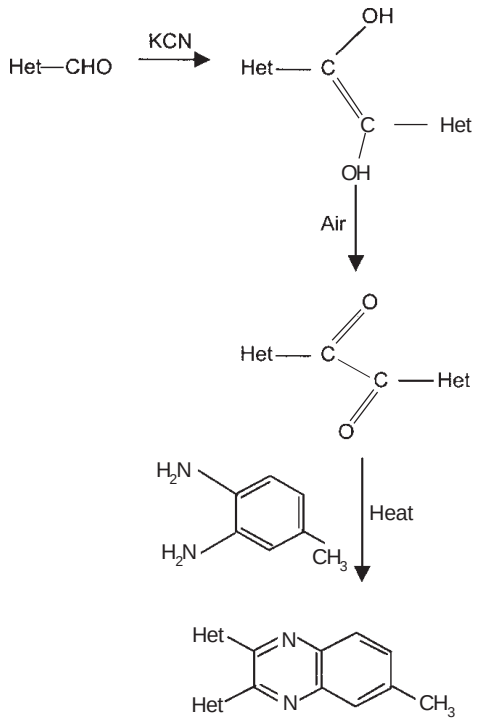
Procedure and analytical data: 2,3-di(quinolyl-2)-6-methyl quinoxaline (1a). To a solution of 2.0 mmole (0.2440 gm) of 4-methyl 1,2-phenylenediamine in 20 ml acetic acid 2.0 mmoles of 1,2-di(quinolyl-2)-1,2 ethanedione (synthesized by the oxidation of 1,2-di-(quinolyl-2)-1,2-ethenediol which was obtained as a result of the condensation reaction of quinoline-2-carboxaldehyde (in the presence of potassium cyanide) was added and refluxed in an oil bath for one hour. The reactive mixture was cooled and 100 ml of water was added into it which resulted in the formation of a beige coloured emulsion which was treated by adding 20% sodium hydroxide solution and the resulting precipitates were filtered. The impure product was then dissolved in alcohol and heated with active charcoal, filtered and reprecipitated with water and crystallized using ethyl alcohol yielding 0.5028 gm (69%), m.p 233.4°C.

IR (Potassium bromide): 3000-3080 (aromatic, = C - H) 2920-3000 (methyl C - H cm^{-1}) ^{1}H NMR (chloroform-D): δ 2.64 (s, CH_3, 3H) 7.41- 8.22 (m, aromatic, 15H).

ms: m/z (relative intensity) 400(M+2,2), 399 (M+1,22) 398 (M+,91), 397(M-1,100), 199(22), 154(2), 128(10), 101(2).

UV (chloroform): λmax 259.6, 399.2 nm.



(1a) Het = (quinolyl-2)
(1b) Het =(6-methylquinolyl-2)

Scheme 1

Elemental analysis: Calculated for $C_{27}H_{18}N_4$; C = 81.38, H = 4.55, N = 14.06 found C = 81.30, H = 4.89, N = 14.19.

2,3-di(6-methylquinolyl-2)-6-methyl quinoxaline (1b). To a solution of 2.0 mmole (0.2440 gm) of 4-methyl 1, 2-phenylendiamine in 20 ml acetic acid, 2.0 mmoles of 1,2-di (6-methylquinolyl-2)-1, 2 ethanedione (synthesized by the oxidation of 1, 2-di (6-methylquinolyl-2)-1, 2-ethenediol which was obtained as a result of the condensation reaction of 6-methylquinoline-2-carboxaldehyde (in the presence of potassium cyanide) was added & refluxed in an oil bath for one hour. The reactive mixture was cooled and 100 ml of water was added into it which resulted in the formation of a beige coloured emulsion which was destroyed by adding 20% sodium hydroxide solution and the resulting precipitates were filtered. The impure product was then dissolved in alcohol & heated with active charcoal, filtered and reprecipitated with water and crystallized using ethyl alcohol yielding 0.5030 gm (59%) m.p 152 - 3°C (Scheme 1).

IR (Potassium bromide): 2980-3070 (aromatic, = C - H) 2870-2980 (methyl C-H) cm^{-1} 1350 and 1430 (methyl, asymmetric and symmetric C-H).

^{1}H NMR (chloroform-D): δ2.48 (s, $2CH_3$, 3H), 2.65 (s, CH_3, 3H) and 7.32-8.18 (m, aromatic, 13H)

MS: m/z (relative intensity) 428(M^{+2},2), 427(M^{+1},34), 426(M^+,88), 425(M^{-1},100), 411 (M-15,80), 272(20),213(12), 168(2), 167(6), 142(2), 141(4), 115(8).

UV (chloroform): λmax 259.2, 342.4 nm.

Elemental analysis: Calculated for $C_{29}H_{22}N_4$; C = 81.66, H = 5.20, N = 13.13 found C = 81.46, H = 5.33, N = 12.90.

The formation of both quinoxalines i.e., 2,3-di(quinolyl-2)-6-methyl quinoxaline (**1a**) as well as 2,3-di(6-methylquinolyl-2)-6-methyl quinoxaline (**1b**) as a result of condensation reaction between 4-methyl 1,2-phenylenediamine and the respective hetaryl substituted 1,2-ethanedione was confirmed after comparing the UV, IR, ^{1}H NMR and MS spectra of the star-ting materials with that of the synthesized products. Upon observation of the IR spectra of both the hetaryl and methyl substituted quinoxalines the absence of the characteristic C=O absorption band of the 1,2-dicarbonyl compound i.e., in between 1700-1660 cm^{-1} and the bands at 3325 cm^{-1} and 3285 cm^{-1} representative of the symmetric and asymmetric N-H stretching vibrations of NH_2 group of the 4-methyl 1,2-phenylenediamine show the progress of the reaction whereas the presence of the absorption bands at 1585, 1540 and 1480 cm^{-1} due to C=C and C=N bonds of the heterocyclic ring besides the bands between 1440-1420 cm^{-1} due to the symmetric and asymmetric C-H bending vibrations and between 2980-2780 cm^{-1} due to

the stretching vibrations in the spectra of both the compounds confirm the formation of the hetaryl and methyl substituted quinoxalines which was further supported by their ^{1}H NMR spectra. In the mass spectra of both the synthesized compounds, the appearance of a moleculer ion peak at m/z 398 and other expected peaks at 199, 154, 128 and 101 in case of compound (**1a**) whereas a moleculer ion peak at 426 and other expected peaks at 272, 168, 142 and 115 in case of compound (**1b**) confirm the structures of the synthesized compounds.

Key words: Condensation Diketones, Aromatic Diamine

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Short Communication

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Effect of Part Replacement of Silica Sand with Carbon Black on Composite Properties

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Much interest have been shown in the study of filled natural rubber composites (Bristow 1986; Elliot 1986; Barker 1987; Adu and Adeosun 1997; Adeosun *et al* 1999). We have reported the properties of natural rubber filled with locally available materials (Adu *et al* 2000). The effect of local clay, limestone, silica sand and charcoal on the properties of natural rubber has been examined. Results have shown detrimental effects of silica sand on the properties of natural rubber compound. It has been reported that when silica is used as a part for part replacement of carbon black, the heat build up the composite decreased whilst tear resistance improved (Horn 1975; Barbin and Rodgers 1994).

Since the objective of the study is to attempt to find possible industrial utility for the local materials under examination, interest is now focused on possible boosting the reinforcing ability of silica sand by other materials. In the present work, we have replaced silica sand in part with carbon black in natural rubber composites and determined the mechanical and cure properties of the resultant composites. The properties of the composites filled with the mixed silica sand/carbon black were then compared to the properties of composites filled separately with silica sand and carbon black.

Compounding formulation of the composites examined are contained in Table 1. Sample preparation, compounding, curing and the determination of mechanical and cure properties have been described by Adeosun (2000).

Table 1
Compounding formulations

Composites	Parts per hundred rubber (pphr)						
Natural Rubber	100	100	100	100	100	100	100
Zinc Oxide	5	5	5	5	5	5	5
Stearic acid	3	3	3	3	3	3	3
Mbt [(a)]	1	1	1	1	1	1	1
Suplhur	3	3	3	3	3	3	3
Carbon black	-	10	20	25	30	35	50
Silica sand	50	40	30	25	20	15	-

(a) = Mercaptobenzothiazole (accelerator)

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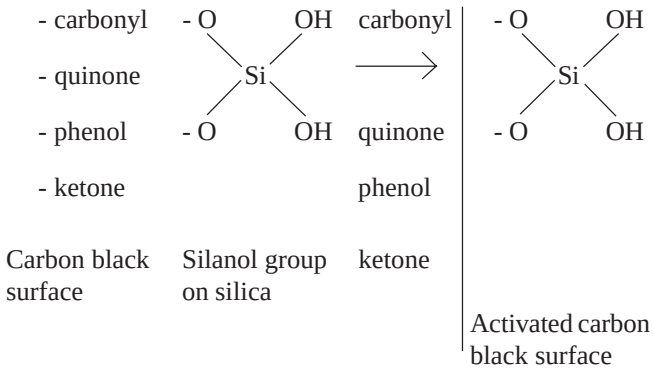
The mechanical properties (tensile strength, modulus and elongation at break, hardness, resilience and hysteresis) and cure properties (scorch time, cure time, torque rise, cure rate index, crosslink density and reversion resistance) have been determined and are presented in Figs. 1-5.

It is observed that the composite loaded (50 pphr) with silica sand showed a strength of 1.14KNm^{-2} which improved tremendously on the replacement of 10pphr with carbon black. (Fig 1). The tensile strength increased progressively with increasing carbon black content in the rubber composite matrix. It has been known for some time that the use of silica in rubber compounds is problematic mainly due to the presence of siloxane and silanol groups on the surface of the filler (Wolff *et al* 1994). The silanol or hydroxyl groups have been reported to be acidic in nature (Hair and Hertl 1970). The analysis of dry carbon black surface indicated the presence of a range of hydrocarbon groups, which are capable of reacting with other functional groups (Bras and Papirer 1979; Barbin and Rodgers 1994). The enhancement of tensile strength of silica sand by carbon black can therefore, be suggested to be as a result of reaction of such groups as carbonyl, lac quinone, phenol and ketone on the carbon black surface with the silanol and hydroxyl groups of the silica sand leading to the formation of covalent bond with the carbon black.



In addition to this type of reaction, Le Bras (1979) has demonstrated that carbonyl, phenolic, quinone and other functional groups on the carbon black surface react with the natural rubber polymer. He provided evidence that chemical crosslinks exist between these materials in vulcanizates. Such chemical crosslinks can probably contribute to the enhancement of the energy properties of the composite such as tensile strength and modulus.

The modulus of the composite loaded with silica sand only showed a magnitude of 0.73KNm^{-2} which is more than tripled

on the replacement of 10 pphr of silica sand with carbon black (Fig 1). Modulus increases with increasing carbon black content in the composite mix. The explanation for this enhancement could be found in the suggested reactions between carbon black/silica-sand and carbon black/polymer within the composite matrix. The elongation at break (E_B) generally decreased with increasing carbon black content in the mix. This is expected because the brittleness of the composites is known to increase with decreasing strength.

The hardness of the composites loaded with mixed filler (silica sand/carbon black) showed a relatively superior values to that of the composite loaded singly with silica sand (Fig 2). The resilience of these composites (loaded with mixed fillers) however showed inferiority to that of the composite loaded singly with silica sand (Fig 2). The hysteresis also showed higher magnitude for the mixed fillers compared to silica sand within the concentration range of fillers used in the present work (Fig 3).

The scorch time which is the time between the beginning of the heat cycle to the time curing begins, measures the processing safety period of the composite. The scorch time (Fig 4) of the composite loaded with silica sand only showed a magnitude of 28.8sec. Which decreased on the replacement of 10pphr of silica sand with carbon black. The scorch times of composites loaded with the mixed fillers showed comparable values similar to that composite loaded with carbon black alone. The present results indicate that the composite loaded with carbon black alone and the mixed fillers have inferior processing safety period compared to the composite loaded with silica sand alone. The cure time (Fig 4) followed the same trend as the scorch time but with different implication. The composites loaded with mixed fillers cures faster than the composites loaded singly with silica sand or carbon black. The composite loaded with carbon black has a faster curing than the composite loaded with silica sand. Long cure times and slow cure tares have been reported for silica

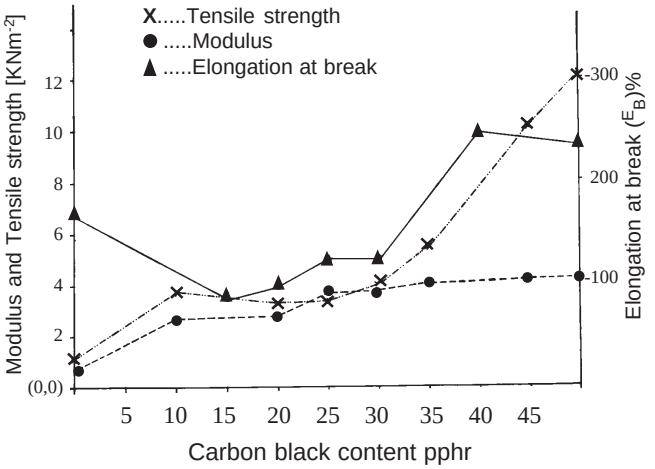


Fig 1. Plots of Tensile strength, modulus and elongation at break versus filler content in the mix.

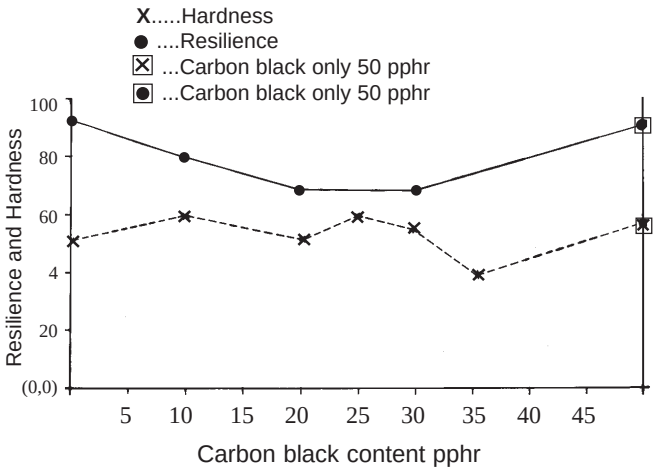


Fig 2. Plot of resilience and hardness versus carbon black content in the mix.

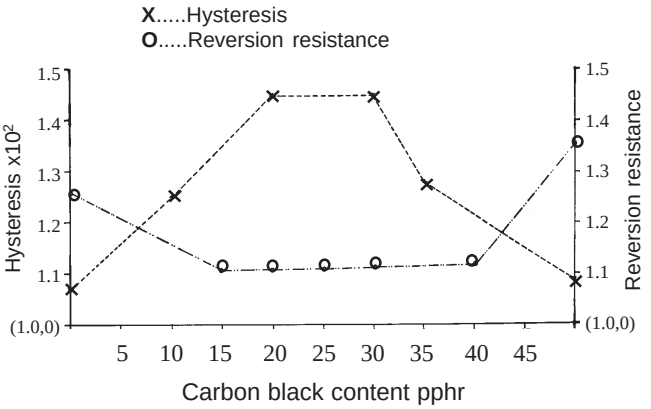


Fig 3. Plot of hysteresis and reversion resistance versus carbon black content in the mix.

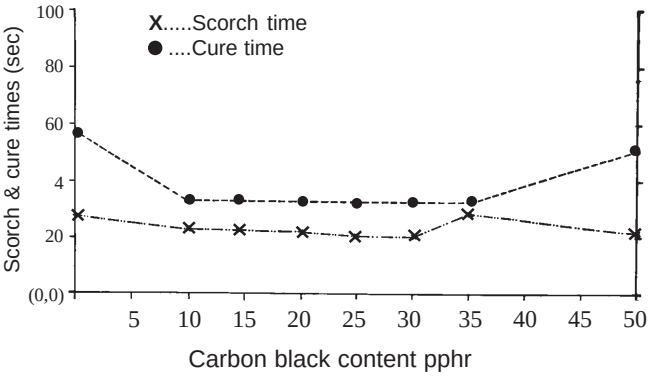


Fig 4. Plots of cure and scorch times versus carbon black content in the mix.

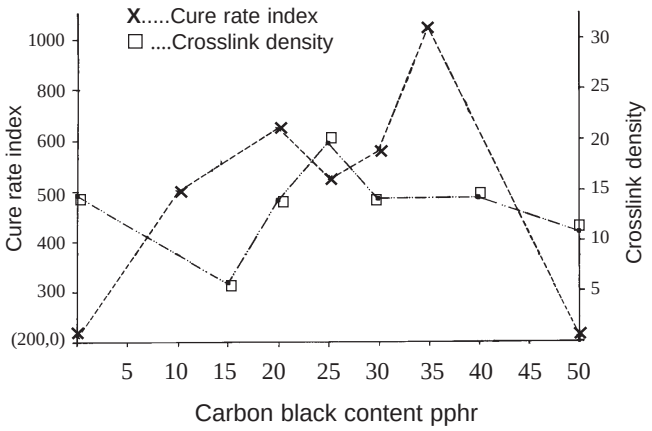


Fig 5. Plots of cure rate index and crosslink density versus carbon black content in the mix.

loaded rubber compound (Wolff *et al* 1994). This observed behaviour has been explained to be due to the strong tendency of the silica filler, (which is polar and hydrophilic) to absorb moisture (Hockley and Pethica 1961; Basset *et al* 1968). The amount of water adsorbed on the surface of the filler controls the ionization of the hydroxyl groups (Wagner 1976), resulting in detrimental effects on the curing attributes of compounds.

The cure rate index of the composites loaded with silica sand or carbon black showed slow cure rates compared to the composites loaded with mixed silica sand/carbon black (Fig 5). The reason adduced for the long cure time of silica sand explains why the cure rate of composite loaded with silica sand is slow. The crosslink density (Fig 5) of the composite loaded with carbon black or with mixed silica sand/carbon black showed superiority (except the composite filler of ratio 3:7) to that loaded with silica sand alone. This might be due to the presence of carbon atoms in carbon black filler, which would be made readily available for crosslink purposes. The reversion resistance deceased on the replacement of 10pphr of silica sand with carbon black as depicted in Fig 3, remained fairly unaffected by further replacement of silica sand with carbon black.

Results revealed that within the filler content range used in the present work, the hardness, modulus, and tensile strength of composites loaded with silica sand/carbon black showed enhanced magnitude over the composite loaded singly with silica sand. These parameters generally increased with increasing carbon black content in the composite. New area of use requiring moderate level of tensile strength, hardness and modulus (as in soles of shoes and engine mounts) is therefore opened up for silica sand.

***Key words*:** Silica sand, Carbon black, Cure properties, Mechanical properties, Natural rubber composite, Filler loading.

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STUDIES ON THE PRODUCTION OF SPORE CRYSTAL BY *BACILLUS THURINGIENSIS* CAMB 3-023 IN THE STIRRED FERMENTOR

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The insect pathogen *Bacillus thuringiensis* CAMB 3-023 was cultured using CSL-salt medium, in the stirred fermentor of 14 l capacity by batch process. The rates of aeration and agitation were kept at l/l/min and 200-300 rpm respectively. The sporulation hence in crystal protein synthesis was faster and much improved in comparison with shake flasks. It reached maximum ($4.2x10^9$/ml) in 48 h after inoculation with vegetative inoculum (10% v/v) developed in shake flasks. The bacterium was grown in the presence of different amounts of CSL (2.0-4.0% w/v). The optimum level of CSL was found to be 2.0% w/v. The addition of $MnSO_4$ and $CaCl_2$ during the course of fermentation enhanced sporulation.

Key words**:** *Bacillus thuringiensis*, Spore crystal protein, Fermentation.

Introduction

The bioinsecticide preparations of *Bacillus thuringiensis* are of great economic importance owing to their lethal effect against a wide variety of Lepidoptera, Diptera and Coleoptera larvae (Jamnback 1973; Scherrer *et al* 1977; Aronson *et al* 1986; Hofte and Whiteley 1989; Karim *et al* 1999a). The production of bioinsecticide is basically a cell biomass production process, provided the nutritional requirements of the bacterium for sporulation and endotoxin formation is fulfilled. Extensive studies have been carried out in connection with the nutritional requirements for growth and sporulation by submerged fermentation in shake flasks (Kang *et al* 1992; Zafar and Riazuddin 2002) and stirred fermentors (Goldberg *et al* 1980; Mohd-Salleh and Beegle 1980; Smith 1982, Kang *et al* 1992). The use of carbon sources such as glucose, a nitrogen source like yeast extract and the presence of potasium salts, manganese and some micronutrients are essential. Yeast extract is a source of amino acids, vitamins and minerals and the strain employed does not require vitamins as reported by the workers (Nickerson and Bulla 1974; Arcas and Yantorno 1987, Zafar *et al* 2001). Production of crystal protein in the bacterial cell follows the end of exponential growth phase and involves the assembly of proteins synthesized *de novo* at the beginning of sporulation (Scherrer *et al* 1973; Goldberg and Margalit 1977). Fed-Batch culture method has also been employed as a means of increasing cell mass or product concentration (Kang *et al* 1992). One reasonable way to increase spore production is to achieve high cell biomass and subsequently allow sporulation to occur. The spore concentrations obtained during different studies were $6.5x10^7$ (Pearson and Ward 1988), $1.8x10^8$ (Zafar and Riazuddin 2002a), $2.1x10^9$ (Arcas *et al* 1984) and $1.25x10^{10}$ per ml (Kang *et al* 1992) by using fed-batch culture method.

In continuation of our studies in shake flasks (Zafar and Riazuddin 2002a) the present work describes the propagation of a local isolate of *Bacillus thuringiensis* CAMB 3-023 (Karim *et al* 1999a) in the stirred fermentor for sporulation by batch process. Corn steep liqour (CSL) was used as a basal medium. The parameters such as concentration of CSL, agitation rate, size of inoculum, fermentation time for sporulation, dry pellet mass and crystal protein yield during fermentation were optimized.

Materials and Methods

Sources of raw materials/chemicals: Corn steep liquor (C.P.C. Rafhan Industries, Faisalabad, Pakistan), silicon based antifoam 30% (Sigma, Cat.No.A5758), Acetone (Merk, Cat.No.822251), $MnSO_4$ (Riedel-De Haenag,Cat.No.OE406-24) and $CaCl_2$ (Merk, Cat.No.102820).

Organism: The bacterium used was locally isolated *Bacillus thuringiensis* CAMB 3-023, carrying CryIA(a)/ IA(c) genes, previously characterized through molecular analysis according to the nomenclature of Bt. pesticidal crystal protein genes. It was maintained on Luria agar slants consisting of (g/l). Tryptone 10.0; Yeast extract, 5.0, NaCl, 5.0 and Difco Agar 15.0, pH 7.5. The cultures were incubated at 30˚C for 72 h and then stored at 4˚C. All the media, otherwise stated, were sterilized at 121˚C for 15 min.

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Inoculum preparation: The vegetative inoculum for fermentor studies was developed step wise in two stages: (i) First inoculum stage: 50ml LB medium, contained in 250ml cotton wool plugged conical flask, was inoculated by transferring a loopful of cells from LB agar slants. The flasks were rotated at 200 rpm using New Brunswick Incubator Shaker at 30°C for 24 h. (ii) Second inoculum stage: 500ml of 2.5% CSL medium in 2 liter flask was inoculated by transferring Ist inoculum and incubated for 24 h as mentioned above. Vegetative growth/sporulation by using Gram's staining and spore staining reactions were observed throughout the process of fermentation.

Fermentation: The fermentation studies were carried out in a 14 l New Brunswick Microferm Fermentor with working volume of 10 l. The fermentor containing CSL medium, with initial pH 7.0, adjusted by using NaOH solution (10.0%) was placed in the autoclave and sterilized at 121°C for 45 min. The size of the inoculum was kept at 10% v/v unless otherwise stated. The rates of agitation and aeration were 200-300 RPM and l/l/min, respectively. The foaming during fermentation was controlled manually by adding silicon based antifoam (30%) drop wise. The pH was recorded by pH probe and the sporulation of cells, however, was determined by microscopy during the course of fermentation. Finally, the number of spores/ml was estimated by colony forming unit assay. The solution containing $MnSO_4$ and $CaCl_2$ were added to the fermentor, 24 h after inoculation for the enhancement of sporulation. At the end of fermentation, the bacterial cell mass was harvested at room temperature by centrifugation (Beckman, Model J2-21) at 8000 rpm for 10-15 min. The wet pellet was treated with acetone to remove water and dried in a hot air oven at 50°C for 10 h. The dried cell mass was powdered using electric grinding jar mill to the mesh size 200 and formulation of bioinsecticide was developed for field studies (Karim *et al* 1999a; Zafar *et al* 2000; Karim *et al* 2000; Rehman *et al* 2002; Zafar *et al* 2002b).

Analysis: Colony forming units (CFU) of heat resistant spores were determined by making serial dilution of fermented culture, after giving heat shock at 80°C for 10 min. The samples were spreaded on LB agar plates and incubated at 30°C for overnight. The crystal protein concentration was estimated by dissolving known amount of dried cell mass in solublizing buffer (50mM sodium carbonate, 10mM Dithiothreitol, pH 10) and incubated at 37°C for 4-6 h with frequent shaking. The crystal protein in the suspension was solubilized at alkaline pH; insoluble fraction and cellular proteins were removed by centrifugation at 14,000 rpm for 10 min at room temperature. Protein concentration was measured by the method of Bradford (1976).

Results and Discussion

Corn steep liquor and byproduct of starch/glucose industries were found to be complete medium providing adequate amounts of all nutrients for the propagation of locally isolated *Bacillus thuringiensis* CAMB 3-023 instead of highly expensive raw materials such as yeast extract, peptone and casein hydrolysate.

Effect of the concentration of CSL (1.0-4.0% w/v) on the production of spore/ml, dry mass and crystal protein yield of *Bacillus thuringiensis* CAMB 3-023 was carried out in the stirred fermentor. The addition of $MnSO_4$ and $CaCl_2$ salts in CSL medium increased the yield substantially. For *Bacillus thuringiensis* propagation, it is necessary to use an adequate

Table 1

Effect of the concentration of CSL with and without salts on the production of spores, dry cell mass and crystal protein yeild of Camb Bt. 3-023 in the stirred fermentor*

CSL% (w/v)	Addition of salts**	Replicates	Sporulation spores/ml	Dry mass g/1	Crystal protein mg/g dry mass
1.0	–	2	$3.0x10^6$	1.5	100.0
	+	2	$3.0x10^6$	1.6	104.4
2.0	–	3	$3.5x10^7$	2.5	119.0
	+	4	$0.5x10^8$	3.0	159.0
2.5	–	3	$2.8x10^7$	2.6	105.3
	+	3	$4.3x10^7$	3.2	111.0
3.0	–	2	$5.0x10^6$	4.0	68.2
	+	3	$9.5x10^6$	4.4	72.0
4.0	–	3	$1.3x10^5$	4.9	47.4

* Agitation 200 RPM for 70 h cultivation ; ** $MnSO_4$ + $CaCl_2$

Table 2
Effect of agitaton on the production of spores, dry cell mass and crystal protein yield of Camb BT 3-023 in the stirred fermentor*

CSL** % (w/v)	Agitation RPM	Replicates	Sporulation spores/ml	Dry mass g/l	Crystal protein mg/g dry mass
2.0	200	2	$2.3x10^7$	2.7	106.4
	300	5	$1.4x10^9$	3.0	183.0
2.5	200	3	$0.6x10^7$	3.0	100.0
	300	3	$2.2x10^8$	3.4	154.7
3.0	200	2	$3.9x10^6$	3.4	54.4
	300	2	$0.2x10^8$	3.5	112.5

* Cultivation 48 h; ** CSL medium with salts

organic carbon and nitrogen source (Arcas and Yantorno 1987). Although CSL seems to be the most convenient source of carbon and nitrogen, the ratio of carbon and nitrogen source in the medium is very critical for the optimum sporulation. With the increase of CSL concentration in the medium, the rate of sporulation and crystal protein yield was decreased (Table 1). The maximum sporulation was observed in 2% CSL medium with $MnSO_4$ and $CaCl_2$ salts, at 30-32 h after inoculation. The cells were harvested for 68-70 h after inoculation. The maximum vegetative growth took place within first 32 hours and by the addition of salts at this point, the sporulation was $0.5x10^8$ per ml and crystal protein yield was 159 mg/g dry cell mass.

The adequate supply of dissolved oxygen to the culture is highly critical for maximum cell biomass formation. The supply of oxygen to the culture depends on both the rates of aeration and agitation during the course of fermentation. The rate of agitation was changed from 200 RPM to 300 RPM while the aeration was kept at l/l/min and it greatly enhanced both the sporulation and crystal protein yield. Maximum sporulation was observed 48 h after inoculation. The maximum vegetative cell growth reached within first 20-22 hrs and addition of salts was made at this point. In 2% CSL medium at 200 RPM, the rate of sporulation after 48 h fermentation is $2.3x10^7$ with the crystal protein yield of 106.4 mg/g dry mass. By increasing agitation upto 300 RPM, the maximum sporulation ($1.4x10^9$ spores/ml) was observed with the crystal protein yield of 183 mg/g dry mass (Table 2) whereas at 200 RPM the maximum sporulation/crystal protein was acheived in the same medium at 70 hrs fermentaiton after inoculation (Table 1).

Thus the CSL (2% w/v) medium containing salts was more effective in terms of spore/crystal protein yields, produced in 48 hrs fermentation at 300 RPM agitation rate with the aeration of l/l/min.

Acknowledgement

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RELATIONSHIPS BETWEEN HEAVY METAL CONTENT AND BODY WEIGHT OF SELECTED FRESHWATER FISH SPECIES OF THE LOWER IKPOBA RIVER IN BENIN CITY, NIGERIA

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The relationships between the heavy metals content and body weight of edible muscle of selected freshwater fish species of the lower Ikpoba river in Benin City Nigeria, were studied using the atomic absorption spectrophotometry technique. 27 samples of *Clarias gariepinus* (average wt: 110.80 ± 36.31g); 36 samples of *Channa obscura* (average wt: 65.20 ± 27.20g) and 18 samples of *Chromidotilapia guentheri* (average wt: 80.10 ± 31.70g) were used for the study. Mean heavy metals concentration (µg/g dry wt.) varied from Cd (0.02 ± 0.01), through Cu (0.25±0.07), Pb (0.40 ± 0.60) to Zn (6.0 ± 1.03) in *Clarias gariepinus* and through Cd (0.02 ± 0.03), Cu (0.04 ± 0.02), Pb (0.27 ± 0.05) to Zn (2.10 ± 0.02) in *Channa obscura* and through Cd (0.02 ± 0.05), Cu (0.30 ± 0.05), Pb (0.40 ± 0.2) to Zn (6.15 ± 1.70) in *Chromidotilapia guentheri*. Relatively low metal values were recorded in *Channa obscura*. Significant correlations were recorded between cadmium, lead, zinc and copper and body weight of *Clarias gariepinus* and *Chromidotilapia guentheri* ($P < 0.05$). However, in the predatory *Channa obscura*, statistical analysis showed that copper and zinc contents were significantly correlated while cadmium and lead were not ($P > 0.05$).

***Key words*:** Heavy metal, Body weight, Fish muscle, Lower Ikpoba river, Benin City, Nigeria.

Introduction

Freshwater organisms including fish have the ability to concentrate heavy metals in their tissues to the concentration levels which are comprised of several orders of magnitude higher than those in water and sediment (Perttila *et al* 1982; Kiorboe *et al* 1983). The accumulation and biomagnification of heavy metal in the tissues of animals have recently received considerable attention (Deb and Santra 1997). Heavy metals accumulated in the organs of various organisms may be speedly transferred from the surrounding environment into the food chain (Adema *et al* 1972). Younger fishes tend to accumulate more metals which tend to decrease with the age of the fish (Benson *et al* 1976). A close relationships between the heavy metals content with age and size with some freshwater organisms such as *Mytilus edulis* were established by NAS (1980). These organisms including fish serve as bioindicators of heavy metal pollution in the aquatic environment.

A number of studies on heavy metal concentration in whole fish and fish organs in Nigerian waters were reported by several researchers including (Kakulu *et al* 1987; Fodeke *et al* 1989; Sadik 1990; Ezemonye 1992; Fufeyin 1994). Oguzie (1996) reported relatively high metal levels in some ubiquitous fish species of the lower Ikpoba river in Benin, even though the metal values were lower than values recommended in fish and fishery products by FAO (Nauen 1983). Neither of these studies reported the relationship between the heavy metal content and body weight of fish of different trophic levels especially those of inland water bodies in Nigeria such as the Ikpoba river in Benin City. This paper reports the correlations among cadmium, lead, zinc and copper content and body weight of selected freshwater fish species of commercial importance in the lower Ikpoba river in Benin City, Nigeria.

Materials and Methods

Study location. The main features of the study area including the sampled zones had earlier been described by Oguzie (1999) and are presented in Fig 1.

Sample collection and preparation. Fish samples were collected between April 1991 and June 1992 by means of gill nets, cast nets and baited hooks at varying depths of the lower Ikpoba river. The specimens used for the study included 27 *Clarias gariepinus* (Teugels) (18.60-110.80g); 36 samples of *Channa obscura* (Gunther) (12.50-650.20g) and 18 *Chromidotilapia guentheri* (Sauvage) (10.5-80.10g). Fish lengths (cm) and fresh weights (g) were determined with a measuring board and a top loader (Mettler PE.360) respectively. The edible muscle tissue of each fish (~100g) was obtained by dissection and placed in petri-dishes. They were oven dried

at 80˚C for 24 h. Each dried sample was weighed and milled separately by means of a porcelain mortar. They were kept in labelled plastic packs, sealed and stored prior to analysis.

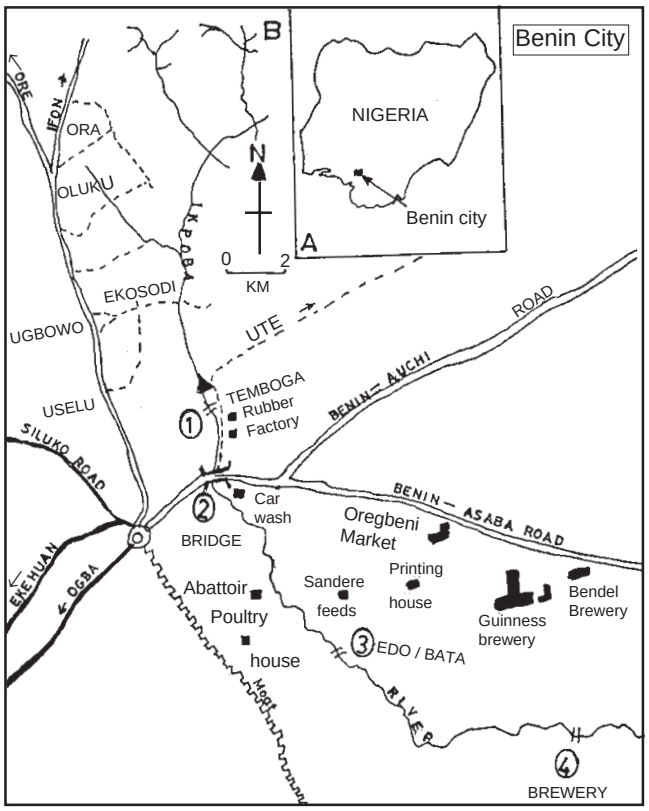
Digestion and analysis. Edible muscle samples were digested using the organic extraction technique described by Sreedevi *et al* (1992).

1g sample was in each case placed in a 50ml Kjeldahl flask. 10ml nitric acid, 2ml perchloric acid and 2ml sulphuric acid (5:1:1 ratio) were respectively added to muscle samples in the flask. Contents of each flask were digested with moderate heat under a hood. Digestion was terminated with the appearance of white fumes. Digestates were diluted to 10ml with distilled water and further boiled for a few minutes. They were allowed to cool and subsequently filtered into 50ml volumetric flasks. A Varian Techtron Spectr AA 10 (model 65) Atomic Absorption Spectrophotometer was used for heavy metal analysis by the method previously described by Oguzie (1996). Triplicate samples were analysed. Standard and blank samples were run with each set of experiments. The correlation coefficient analysis and its statistical tests followed the method of Alder and Roessler (1972).

Results and Discussion

Presented in Table 1 are mean concentration values (mg/g dry wt.) of heavy metals in the edible muscle of *Clarias gariepinus, Channa obscura* and *Chromidotilapia guentheri*, while, Figs 2, 3 and 4 show the relationships between the metal contents in the muscle tissue and body weight of the fish species.

Based on the data presented, the range for Cd content varied from 0.01 to 0.08µg/g with highest value (0.08 µg/g) recorded in *C.guentheri*. A uniform mean Cd value (0.02µg/g) was recorded in the three fish species studied. Pb content varied from 0.20µg/g in *C.guentheri* to 1.25µg/g in *C.gariepinus* with lowest mean value (0.27±0.05 µg/g) in *C.obscura*. The range for Zn varied from 2.30µg/g in *C.obscura* to 8.15µg/g



Source Federal Surveys Nigeria 1964

Fig 1. The study area; A. Nigeria showing Benin city; B. The study river showing the four samplimg zones.

Table 1
Concentration values (µg/g dry wt.) of heavy metals in the edible muscle of selected fish species of the lower Ikpoba river

Species	Average wt. g	No of samples	Heavy Metals Cd	Cu	Pb	Zn
Clarias gariepinus	110.80g ±36.31g	27	0.01-0.06[a] 0.02[b]±0.01	0.09-0.35 0.25±0.07	0.30 - 1.25 0.40±0.60	5.30 - 7.50 6.00±1.03
Channa obscura	65.20g ±27.20g	36	0.01-0.05 0.02±0.03	0.02-0.92 0.04±0.02	0.25-0.40 0.27±0.05	2.30 - 2.90 2.10±0.02
Chromidotilapia guentheri	80.10g ±31.70g	18	0.01-0.08 0.02±0.02	0.15-2.15 0.30±0.05	0.20 - 00.50 0.40±0.02	4.25 - 8.15 6.15±1.70

N.B. a, range; b, mean ± S.D.

in *C.guentheri* with lowest mean value (2.10±0.02µg/g) in *C.obscura,* while Cu content varied from 0.02µg/g to 2.15 µg/g with highest mean value (0.30±0.05 µg/g) in *C.guentheri*.

The correlation coefficients between cadmium, lead and zinc and body weight for *C. gariepinus* (Fig 2) were 0.75, 0.58 and 0.73 respectively. These values were significantly correlated ($P < 0.05$). Though the correlation coefficient for copper, was low (0.29), the value was also significantly correlated ($P < 0.05$). The correlation coefficients of cadmium, copper, lead and zinc for *C. guentheri* (Fig 3) were 0.61, 0.85, 0.84 and 0.83, respectively. The values were all significantly correlated ($P < 0.05$). Though poor correlation coefficients were recorded for *C. obscura*, statistical analysis, however showed that copper and zinc contents were significantly correlated while cadmium and lead were not ($P > 0.05$) (Fig 4).

The heavy metal content in the muscle tissue of fish might be related to the feeding habits and distribution pattern of the fish. Metal uptake in fish was reported to occur through absorption across the gill surface or through the digestive tract during food intake (Brooks and Rumsby 1974).

The low metal concentration recorded in the muscle tissue of *C. obscura* may be attributable to its food habits being a

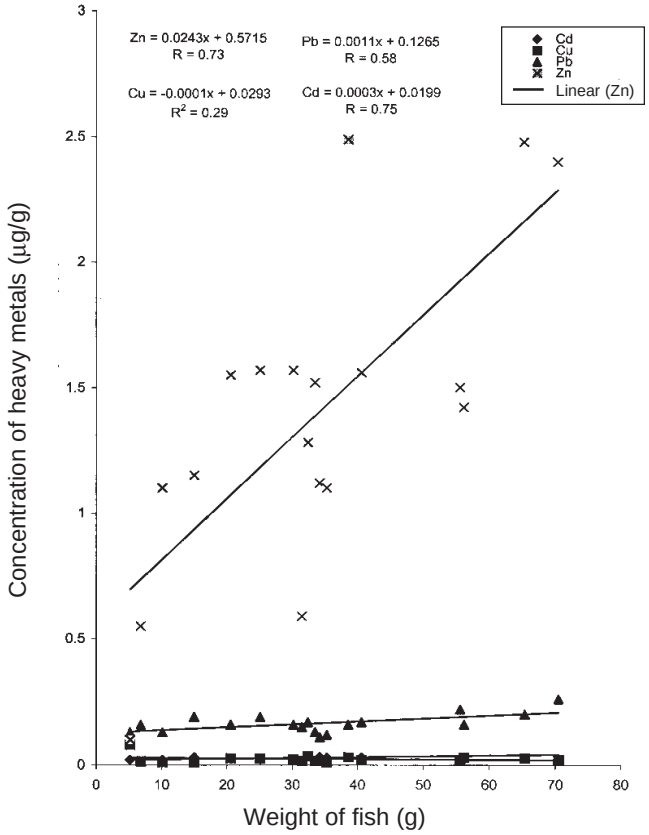


Fig 2. Heavy metals concentration - Body weight relationships of *Clarias gariepinus*.

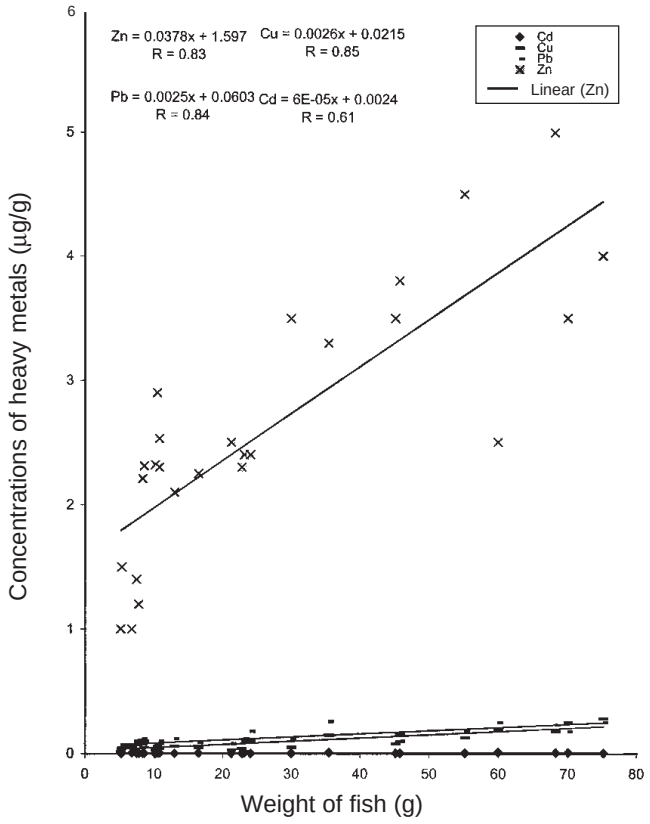


Fig 3. Heavy metals concentration - Body weight relationships of *Chromidotilapia guentheri.*

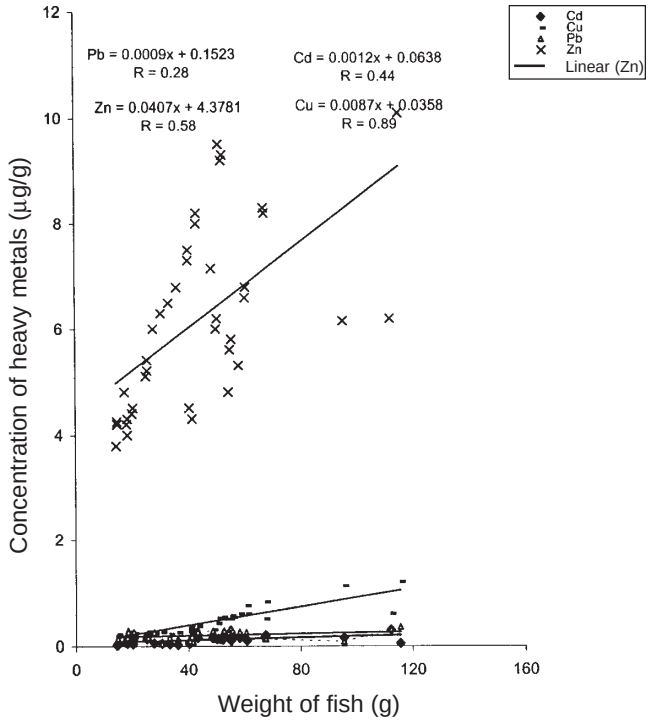


Fig 4. Heavy metals concentration - Body weight relationships of *Channa obscura*.

pre-datory fish that is reported to stalk its prey in the open water column (Reed *et al* 1967). The heavy metal contents in the fish muscle tissue arising from food intake may be able to reflect the level of metal pollution of the lower Ikpoba river probably due to the distribution pattern of this predatory fish species previously reported by Oguzie (1996).

C. gariepinus and *C. guentheri* are considered bottom dwelling species. They occasionally plunge their head deep between plant roots where they take up load of sediment (rich in heavy metals) from which they sift out and consume edible particles including worms, insect larvae and materials of plant origin (Reed *et al* 1967). This report clearly suggests that based on their trophic status, the heavy metal content of their muscle tissues arising from food intake would probably reflect the corresponding heavy metal concentrations of the lower Ikpoba river.

Bottom sediment, which Nishida *et al* (1982) described as "bank" for heavy metals invariably contain relatively high levels of the various food items consumed by these omnivorous and detritivorous fish species which tend to change their feeding niche when the preferred food is in short supply. Since it was reported (Reed *et al* 1967) that these fish species visit the bottom sediments frequently in search of food, hence it is logical to infer that they will concentrate relatively high levels of heavy metals in their muscle tissues.

Studies on trophic relationships among fish species by Lowe-McConnell (1975) show that the ability to use many different foods effectively is an important characteristic of ubiquitous species which have better chance than specialists of becoming widely distributed and which could be effectively used as bioindicators.

The close relationships between the heavy metals content and body weight of *C. gariepinus* and *C. guentheri* and the corresponding poor relationships of *C. obscura* seems to support the view that trophic status and distribution pattern play important role in the accumulation of heavy metals in fish tissues in the aquatic environment.

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CYSTIC ECHINOCOCCOSIS IN DOMESTIC RUMINANTS IN COX'S BAZAR OF BANGLADESH

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This investigation was carried out to describe the local epidemiological pattern of cystic echinococcosis among the indigenous domestic ruminants in the Cox's Bazar district of Bangladesh. Home visits and family interviews revealed several socio-economic and cultural characteristics which are thought to be involved in the transmission cycle and widespread occurrence of cystic echinococcosis in the area. Fecal examination of stray and house dogs showed high infection levels (50.65%) with *Echinococcus granulosus.* A significantly higher ($p < 0.00$) prevalence of cystic echinococcosis was recorded in sheep (52.11%) than buffaloes (36.11%), cattle (30.62%) and goats (14.73%). The values of chi-square test and regression-coefficient analysis strongly indicate that age is an important risk factor of being higher infestation with cystic echinococcosis. While sex had no influence on the prevalence of the disease. Lungs of all ruminant hosts were the most common predilection site for *Echinococcus* cysts, liver ranked second, followed by spleen, kidneys and heart. Fertility rate of the cysts was recorded high in sheep (65.43%) than buffaloes (16.33%), goats (14.80%) and cattle (8.11%). The high level of infection with cystic echinococcosis in domestic ruminants and dogs suggests that there is an urgent need for control measure against the disease in the Cox's Bazar area.

***Key words*:** Cystic echinococcosis, Prevalence, Local epidemiology, Domestic ruminants, Cox's Bazar.

Introduction

Cystic echinococcosis, caused by the larval stage of *Echinococcus granulosus*, is an important parasitic disease of domestic herbivorous animals and man. It occurs in all major continents of the world and is particularly important in developing countries where many rural inhabitants live under poor sanitary conditions and in close proximity to their pets and domestic animals (Anderson 1997). The public health and economic impacts of cystic echinococcosis are enormous in terms of morbidity and mortality in humans and losses due to reduced productivity and condemnation of infected edible offal in animals. Although, few scattered reports on the occurrence of echinococcosis/hydatidosis are available in animals (Islam 1981, 1982 a,b; Karim *et al* 1982) and man (Khan 1990), however, no organized approach has yet been made to identify and quantify the biology and epidemiology of the disease in Bangladesh. This investigation was therefore undertaken to describe the prevalence and distribution of cystic echinococcosis and also, to determine the socioeconomic and cultural factors that may govern the local epidemiological pattern of the disease in a particular geographic area. For this, Cox's Bazar district, which is located in the southeastern part of Bangladesh was included in this study. The Cox's Bazar is a healthy place where the longest sea shore is located and many tourists from home and abroad visit the place. To know the possible health hazards due to cystic echinococcosis to the tourists at risks, Cox's Bazar was therefore selected to render more safety zone for the growing tourism industry.

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Materials and Methods

This investigation was conducted in the Cox's Bazar district, Bangladesh during the period from January, 1995 to June, 1996. To assess the socio-economic and cultural characteristics as well as dog-animal/dog-human association including environmental sanitation, a total of 600 households in the rural and urban areas were interviewed.

A total of 405 cattle, 108 buffaloes, 142 sheep and 292 goats of both sexes and various age groups, slaughtered at different locations were examined randomly. All animals were of local origin and indigenous breed. Carcasses were examined for the presence of echinococcus cysts following the methods described by Islam *et al* (1995). To examine the fertility of the cysts, the brood capsules and daughter cysts were examined for the presence of protoscolices according to the description of Soulsby (1982).

Stastical analysis of the data was performed using Chi-square (X^2) test, Odds ratio (Ψ) and regression-co-efficient analysis as per methods described by Schwabe *et al* (1997), Schlessman (1982) and Lee (1980).

Results and Discussion

Home visits and family interviews revealed that 94% of them have no knowledge about hydatid disease in the Cox's Bazar areas. Of 600 households visited, 144(24%) have house dogs. Stray dogs are most abundant and they frequently come in contact with humans and animals. Dog-animal association was observed in 96% households. Feces of house dogs was disposed only in 5% cases and prophylactic dosing with anthelmintics usually not been practised. Offal of home butchered ruminants was offered to dogs as foods. This study also revealed that the numbers of slaughter houses were inadequate and lack veterinary supervision of the slaughtered animals. Farm animals were grown under traditional management practices. In 79% households, animals were allowed to drink from fecal (humans, dogs and other wild carnivores) contaminated sources (eg., ponds, canals, small ditches, etc). Examination of fecal samples from 77 dogs revealed *E. granulosus* in 39 cases (50.65%).

The detailed results of this investigation are summarized in Tables 1, 2, 3 and 4. This study recorded a significantly higher

Table 1
Distribution of cystic echinococcosis among domestic ruminants in Cox's Bazar

Demographic variables		No. of Animals		Prevalence	X^2 –value	Odds ratio (Ψ)	95% confidence interval for (Ψ) %
Name	Category	Examined	Infected				
	Cattle	405	124	30.62	67.97*		
Animals	Buffaloes	108	39	36.11			
	Sheep	142	74	52.11			
	Goats	292	43	14.73			
	<3 years	71	5	7.04	68.76*		
Age	>3 to <5 years	531	120	22.60			
	>5 years	345	155	44.93			
Sex	Male	523	151	28.87		0.928	0.70, 1.23
	Female	424	129	30.42			

*P<0.00

Table 2
Fitting of logistic regression models for identifying the risk factor

Models	Factors	Regression-Coefficient	S.E	Wald statistic	P-value
Overall	Age	0.4711	0.0468	101.4361	0.0000
	Sex	-0.0906	0.1529	0.3513	0.5534
For Cattle	Age	0.4509	0.0650	48.0486	0.0000
	Sex	-0.0448	0.2345	0.0365	0.8485
For Buffaloes	Age	0.5376	0.1413	14.4714	0.0001
	Sex	-0.0459	0.4419	0.0108	0.9173
For Sheep	Age	0.4876	0.1292	14.0663	0.0002
	Sex	0.0633	0.3631	0.0304	0.8617
For Goats	Age	0.3870	0.1027	14.1947	0.0002
	Sex	0.1893	0.3499	0.2926	0.5886

Table 3
Percentage of *Echinococcus* cyst observed in different organs in domestic ruminants

Species	Organs developed echinococcus cysts					
	Lungs	Liver	Lungs & Liver	Spleen	Kidney	Heart
Cattle	46.77 (58)	40.32 (50)	8.87 (11)	4.03 (5)	-	-
Buffaloes	43.58 (17)	38.46 (15)	10.25 (4)	5.12 (2)	2.56 (1)	-
Sheep	48.64 (36)	33.78 (25)	9.46 (7)	4.05 (3)	2.70 (2)	1.35 (1)
Goats	44.18 (19)	27.09 (12)	11.06 (5)	4.03 (4)	6.97 (3)	-
Overall	46.43 (130)	36.43 (102)	9.64 (27)	5.00 (14)	2.14 (6)	1.35 (1)

Figures in the parentheses indicate number of organs involved

Table 4
Different types of *Echinococcus* cysts recovered from the visceral organs of the domestic ruminants

Species	Types of echinococcus cysts recovered			
	Fertile	Sterile	Suppurative	Calcified
Cattle	12 (08.11)	96 (64.86)	18 (12.16)	22 (14.86)
Buffaloes	8 (16.33)	28 (57.14)	7 (14.29)	6 (12.24)
Sheep	106 (65.43)	32 (19.75)	15 (09.26)	9 (05.56)
Goats	8 (14.80)	36 (66.67)	6 (11.11)	4 (07.41)
Overall	134 (32.45)	192 (46.49)	46 (11.14)	41 (09.93)

Figures in the parentheses expressed as percentage

($p<0.00$) prevalence of cystic echinococcosis in sheep than any other domestic ruminants inspected at Cox's Bazar district. A significantly ($p<0.00$) higher prevalence of cystic echinococcosis was also observed in older animals (>5 years old). The values of Odds ratio and confidence interval did not reveal any significant difference in the prevalence of the disease between males and females (Table1). The logistic regression model fitted for identifying the risk factors revealed a positive association between animals age and disease (Table 2).

The present investigation revealed several important factors which may be considered to understand the local epidemiological patterns of cystic echinococcosis in the Cox's Bazar areas. These include abundance of stray and house dogs, feeding of hydatid infected offal of home butchered farm animals to dogs, poor public health knowledge and environmental hygiene, lack of well run abattoirs, traditional management practices of farm animals, etc. All these factors together might contribute to the high levels of infection with *E. granulosus* in the definitive hosts and increase the environmental contamination with cestode eggs. Under such conditions, the ruminant intermediate hosts get infection readily when grazing along the contaminated road-sides, follow lands and pastures, and drinking water from the contaminated sources. These findings are in conformity with the reports of Schantz *et al* (1995) and Ibrahem and Gusbi (1997).

The high level of *E. granulosus* infection in dogs as recorded in this study reflects the high prevalence of *Echinococcus* cysts in sheep, cattle and buffaloes indicating that these animals play an important role as intermediate hosts. The reason for low prevalence rate of cystic echinococcosis in goats is not clear in this study. Deka and Gour (1998) also reported a higher prevalence rate of the disease in sheep than pigs and goats from India. A significantly lower infection rate in goats has also been reported from Jordan (Abdel - Hafez *et al* 1986; Kamhawi *et al* 1995). However, it is likely that in the absence of sheep and cattle, goats may serve as the principal reservoir host for zoonotic diseases including cystic echinococcosis (Mattossian 1990).

Older animals were prone to develop more *Echinococcus* cysts than growing and young animals. This finding also conforms to the report of Gemmel (1961) and Gusbi (1987). It is thought that the differences in prevalence of cystic echinococcosis may be related to the opportunity for infection and also time taken to developing the metacestodes rather than an increased susceptibility with age. Organ distribution of *Echinococcus* cysts showed that lung is the most common predilection site followed by liver, spleen, kidney and heart irrespective of the ruminant host infected naturally. Our observation found to differ from the reports of Gómez *et al* (1980), Pandey *et al* (1987) and Ibrahem and Gusbi (1997) who demonstrated that liver of sheep, goats and pigs is most commonly infected organ and the lung ranked second, followed by other organs such as kidneys and spleen.

It has been reported that the difference in predilection site may be due to the loose structure of the lungs, which allow a large number of onchospheres to grow more freely in those hosts, in contrast to the more compact tissue of the liver, which may hinder the development of onchospheres (Ibrahem and

Gusbi 1997). Some researchers reported that strain differences may also account for this phenomenon (Ibrahim and Gusbi 1997; Pangi and Ould 1991). Fertility rate of hydatid cysts was recorded significantly high in sheep than other ruminant hosts examined in this study, which indicates that sheep is the more adapted host in the Cox's Bazar areas of Bangladesh. Kachani *et al* (1997) reported that *Echinococcus* cysts are generally more fertile in sheep than in cattle. Further study is required to determine the parasite strains.

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THE FOOD AND FEEDING HABITS OF FISHES OF THE JAMIESON RIVER, NIGERIA

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121 specimens of teleosts comprising of 23 species, 17 genera and 11 families were examined in the study of the ichthyofaunal food habits of the Jamieson River. Cichlidae (34.78%) was richest in species number, and a Bagridae, *Chrysichthys furcatus* (28.93%) was the most numerical species. Analysis of the stomach contents revealed algae, diatoms, macrophytes, zooplankton, shrimps, insects, fish, fish scales, detritus, sand and unidentified material as the main food categories selectively eaten by Jamieson River fishes. The fishes exhibited benthopelagic exploitation and grazing tendency.

Key words: Food and feeding habits, Fishes, Jamieson River.

Introduction

The diet of fishes is a subject of continuing research. Studies on the food and feeding habits of fish continue to attract considerable attention, being the basis for the development of successful capture and culture fisheries management (Lauzanne 1983). The feeding of freshwater fish has been extensively investigated in West Africa particularly in Ghana (Blay 1985) and Nigeria. In Nigeria studies have been performed in Lake Kainji (Imevbore and Bakare 1970; Arawomo 1976; Olatunde 1979); in upper Ogun River (Adebisi 1981); in Lagos and Lekki Lagoons (Fagade 1971; Fagade and Olaniyan 1973) and in a number of rivers in the Niger Delta area (Brown 1985; Umeh 1987; Tetsola 1988; Odum 1992). Information on the feeding habits of fish species will aid the study of trophic relationships (Baijot and Moreau 1997).

In Ethiope River, confluent with Jamieson River, Odum (1992) portrayed the fishes as feeding on a wide spectrum of food organisms from different niches.

Feeding habits of fish species in African flood plain rivers have been reviewed by Welcomme (1979). Lauzanne (1983) also presented a detailed account of the trophic relations of Lake Chad fishes.

Jamieson River is important for commercial fishing and timber raft transportation. The only elaborate fisheries investigations report in this river are on the fish communities entailing diversity, abundance and distribution, also dietary habits of *Brycinus nurse* Ruppel 1832 (Ikomi and Sikoki 1998, 2001). The main objectives of this study are to present species composition and the food and feeding habits of teleost fishes in Jamieson River.

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Materials and Methods

Study Area. River Jamieson is a tributary of Benin River. It is located between Lat 5°54' – 6°00'N and Long 5°41'-5°58'E. The source of the river is at Ugboko-Niro. The river flows southwards through Akuodo, Efurokpe and Oriaja to Sapele Township, where it empties into the Benin River (Fig 1). The entire length is approximately 70km.

The Jamieson River lies in the area with tropical rain forest climate. The rainy season is from March to October. The dry season is from November to February.

The sampling zone for this study is extended from Akuodo through J.A. Thomas Rubber Factory to the Ethiope - Jamieson confluence at Sapele.

It flows through a rain forest vegetation area with characteristic tall trees canopies, effectively shading light from the fringing forest floor. Aquatic floating plants are water lilly *Crinum jagas* and water hyacinth *Eichhornia crassipes*.

Most of the human communities along the bank of the Jamieson River are essentially rural and the freshwater river serves for bathing, washing, commercial fishing, and timber floating and occasional human transport.

The study was conducted from May to July 1995 to determine the species composition and food habits of teleosts. 121 fish specimens were collected with the help of hired fishermen from Akuodo, Efurokpe and Oriaja using cast nets, hook and line. They were put in a cooler containing ice blocks and transported to the laboratory in University of Benin, Nigeria and all of them were identified with guides (Daget and Iltis 1965; Reed *et al* 1967; Holden and Reed 1972). Measurements of each fish were taken of the total length, standard length and weight after mopping peripheral water with filter paper.

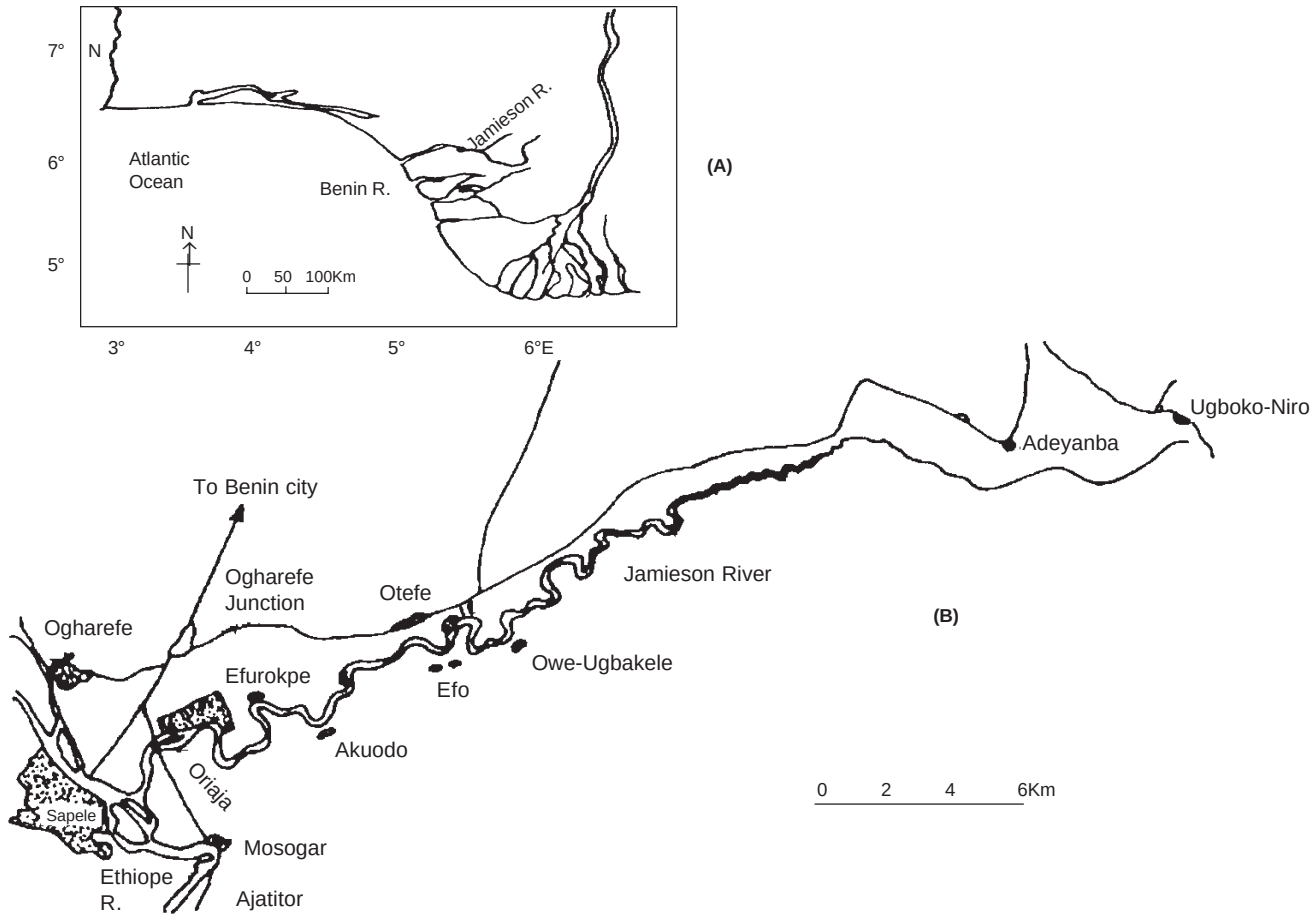


Fig 1. Map of study area showing location (A) and the course (B) of the River Jamieson.

The contents of the stomachs were analyzed to establish the diet of most fishes, but where no definite stomachs exist, the entire gut was inspected.

In the process, the abdomen of each fish was slit open to remove the stomach, further preserved in 4% formalin. Each stomach was in turn slit open, and its contents were emptied into a petri dish.

The macro-organisms were sorted out and the remaining microscopic organisms were examined under a dissecting microscope and a binocular NIKON Compound microscope (Magnification 40 - 100x).

Two methods employed in the analysis of the stomach contents were fullness method and frequency of occurrence.

Fullness method. The fullness of each stomach was assessed using a ranking procedure. The degree of fullness was scored as Empty (0/4), one-quarter (1/4), half (2/4), three-quarter (3/4), and full (4/4) stomachs (Ugwumba *et al* 1990).

Frequency of occurrence method. The number of stomachs of a species in which each type of food item occurred, was listed and expressed as a percentage of the total number of stomachs of that species examined, in order to determine the proportion of the fish population that fed on a particular food item (Hynes 1950). In addition, the relative frequency of occurrence newly used in this paper of each food item category in a fish species was obtained by further expressing the frequency of occurrence (%) as a percentage of the total frequency of occurrence (%) of all the food items categories in the same fish species, and the values were plotted as a bar graph. Also all stomachs in which each type of food item occurred in all fish species were summed up (summation frequency) and expressed as a percentage of the total number of stomachs examined.

Results and Discussion

Species composition. The community of teleost fishes in the Jamieson River is comprised of 23 species, 17 genera and 11 families. They are arranged alphabetically per family.

Bagridae (bagrid catfish) 13.04%; *Auchenoglanis biscutatus* (Geoffrey Saint Hilaire 1827) 2.48%; *Chrysichthys furcatus*

(Gunther 1864), 28.93%; *C. nigrodigitatus* (Lacepede 1803) 2.48%; Channidae (Snakeheads fish) 4.35%; *Channa obscura* (Gurther 1861), 4.13%; Characidae (Characin), 4.35%; *Brycinus longipinnis* (Gunther 1864), 4.13%; Cichlidae *(Cichlid perches)*, 34.78%; *Chromidotilapia guentheri* (Sauvage 1882), 0.83%; (Pelmatochromis) *Hemichromis bimaculatus* (Gill 1862), 4.13%; *H. fasciatus* (Peter 1857), 2.48%; *Pelvicachromis pulcher* (Boulenger 1902), 9.09%; (*Pelmatochromis*); *Tilapia heudelotii macrocephala* (Bleeker 1862), 2.48%; *T. mariae* (Boulenger 1899), 0.83%; *T. melanopleura* (Dumeril 1889), 8.26%; *T. zillii* (Gervais 1849), 8.26%; Clariidae (African mud catfish), 4.35%; *Clarias gariepinus* (Valenciennes 1840). 2.48%; Mochokidae (Mochokid catfish), 8.70%; *Synodontis eupterus* (Boulenger 1901), 4.96%; *S. omias* (Gunther 1864), 0.83%; Mormyridae (Elephant Snout fish), 8.70%; *Hyperopisus bebe occidentalis* (Lacepede 1803), 0.83%; *Petrocephalus bane ansorgii* (Lacepede 1803), 0.83%; Notopteridae (Featherbacks fish), 8.70%; *Papyrocranus afer* (Gunther 1868), 5.79%; *Xenomystus nigri* (Gunther 1868), 2.48%; Phractolaemidae (Blood fish), 4.35%; *Phractolaemus ansorgii* (Boulenger 1901), 1.65%; Polypteridae (Reedfish), 4.35%, *Erpetoichthys calabaricus* (Smith 1865), 4.13%; (*Calamoichthys*) Schilbeidae (schilbeid catfish), 4.35%; *Schilbe mystus* (Linne 1762), 0.83%.

Gut fullness. The results of analysis of stomach contents using the fullness method are shown in Table 1. Of the 121 specimens examined, only 18.18% were empty stomachs, 10.74% were fully loaded stomachs while 23.14%, 16.53% and 31.40% were partly full, 3/4, 1/2 and 1/4, respectively. It is noticeable that among the fish species represented by a single specimen, only a mochokid *Synodontis omias's* stomach alone was empty. The remaining five species characid *Brycinus longipinnis*; cichlids *Chromidotilapia guentheri* and *Tilapia mariae*; mormyrids *Hyperopisus bebe occidentalis* and *Petrocephalus bane ansorgii*, and schilbeid *Schilbe mystus* manifested different degrees of distended stomachs (Table 1).

Table 1
Filled portion of stomachs in Jamieson River fishes

Fish species	Number of specimens	4/4	3/4	1/2	1/4	0
Auchenoglanis biscutatus	3	1	1	1	–	–
Chrysichthys furcatus	35	5	4	5	14	7
C. nigrodigitatus	3	–	1	1	1	–
Channa obscura	5	1	2	–	–	2
Brycinus longipinnis	1	–	1	–	–	–
Chromidotilapia guentheri	1	–	1	–	–	–
Hemichromis bimaculatus	5	1	–	1	3	–
H. fasciatus	3	–	1	1	1	–
Pelvicachromis pulcher	11	1	3	2	3	2
Tilapia macrocephala	3	–	1	1	–	1
T. mariae	1	–	1	–	–	–
T. melanopleura	10	1	4	1	3	1
T. zillii	10	2	1	2	4	1
Clarias gariepinus	3	–	1	1	–	1
Synodontis eupterus	6	–	1	–	3	2
S. omias	1	–	–	–	–	1
Hyperopisus bebe occidentalis	1	–	–	–	1	–
Petrocephalus bane ansorgii	1	–	–	1	–	–
Papyrocranus afer	7	–	3	–	3	1
Xenomystus nigri	3	1	1	–	1	–
Phractolaemus ansorgii	2	–	–	1	–	1
Erpetoichthys calabaricus	5	–	1	1	1	2
Schilbe mystus	1	–	–	1	–	–
Total	121	13	28	20	38	22
%		10.74	23.14	16.53	31.40	18.18

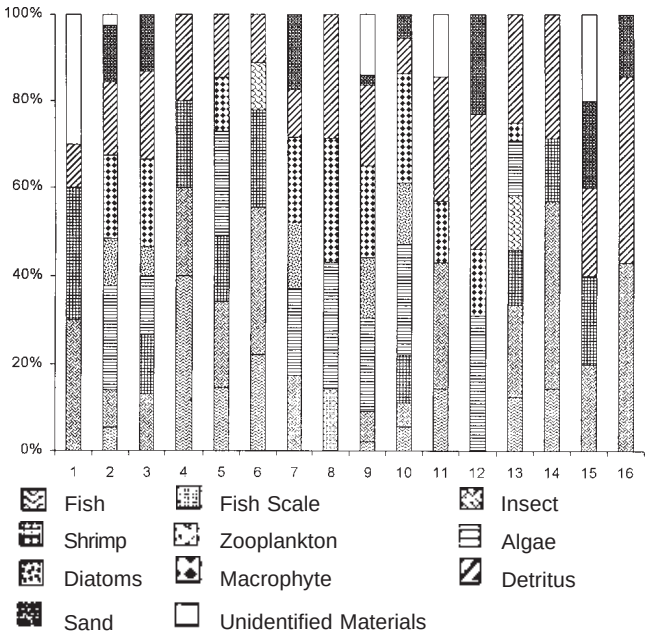
Table 2

The percentage of food items of fish species from Jamieson River

Food items	*A. biscutatus (3)*	*C. furcatus (35)*	*C. nigrodigitatus (3)*	*C. obscura (5)*	*H. bimaculatus (5)*	*H. fasciatus (3)*	*P. pulcher (11)*	*T. macrocephala (3)*	*T. melanopleura (10)*	*T. zillii (10)*	*C. gariepinus (3)*	*S. eupterus (6)*	*P. afer (7)*	*X. nigri (3)*	*P. ansorgii (2)*	*E. calabaricus (5)*
Fish	–	5.31	–	40.00	14.63	22.22	–	–	2.33	5.56	14.28	–	12.50	14.28	–	–
Fish scale	–	–	–	–	–	–	–	14.28	–	–	–	–	–	–	–	–
Insect	30.00	8.85	13.33	20.00	19.51	33.33	17.39		6.98	5.56	28.57		20.83	42.86	20.00	42.86
Shrimp	30.00	–	13.33	20.00	14.63	22.22	–	–	–	11.11	–	–	12.50	14.28	20.00	–
Zooplankton	–	–	–	–	–	11.11	–	–	–	–	–	–	12.50	–	–	–
Algae		23.81	13.33	–	24.39	–	19.57	28.57	20.93	25.00	–	30.77	12.50	–	–	–
Diatoms		10.62	6.67	–	–	–	15.22	–	13.95	13.89	–	–	–	–	–	–
Macrophyte		19.47	20.00	–	12.20	–	19.57	28.57	20.93	25.00	14.28	15.38	4.17	–	–	–
Detritus	10.00	16.81	20.00	20.00	14.63	11.11	10.87	28.57	18.60	8.33	28.57	30.77	25.00	28.57	20.00	42.86
Sand		13.27	13.33	–	–	–	17.39	–	2.33	5.56	–	23.08	–	–	20.00	14.29
Unidentified material	30.00	2.65	–	–	–	–	–	–	13.95	–	14.28	–	–	–	20.00	–

Number in brackets represent species quantity.

The results of stomach content analysis using the frequency of occurrence method were based on 16 fish species, which had two and more specimens. (Fig 2, Table 2). Algae, particularly diatoms, macrophytes, zooplankton, shrimps, insects, fish, fish scales, detritus, sand and unidentified material were the discerned stomach contents categories presumably taken in as food by the fish of Jamieson River. Insects (at larval, nymph and adult stages), shrimps and fish were seen in the guts as entire specimens either at early or advanced stage of digestion. Sometimes disarticulated parts, frequently appendages (antennae, legs, wings), and heads were noticed. The summation frequency of occurrence (%) of the food categories present interesting findings. Employing the summation frequency data, rather than individual species frequency of occurrence only, algae occurred in 60.53% stomachs and macrophytes in 52.63% of the specimens clearly showing that such proportions of the fish examined were herbivores and also able to capture insects (43.86% occurrence) associated with littoral aquatic vegetation and river bottom. The occurrence of diatoms in 27.19% of the stomachs was much higher than zooplankton (3.51%) further pointing to the grazing tendency that detritus and sand occurred in 60.53% and 28.95% of the stomachs respectively, indicatively connotes



1= *Auchenoglanis biscutatus, 2=Chrysichthys furcatus, 3=C. nigrodigitatus, 4=Channa obscura, 5=Hemichromis bimaculatus, 6=H. fasciatus, 7=Pelvicachromis pulcher, 8=Tilapia macrocephala, 9=T. melanopleura, 10=T. zillii, 11=Clarias gariepinus, 12=Synodontis eupterus, 13=Papyrocranus afer, 14=Xenomystus nigri, 15=Phractolaemus ansorgii, 16=Erpetoichthys calabaricus.*

Fig 2. Bar graph showing relative frequency of food items in the stomachs of the River Jamieson teleosts.

bottom feeding habit, such that 16 species obtained some food from the bottom (Fig 2, Table 2). Furthermore, the occurrence of shrimps in 17.54% and fish in 18.42% of the stomachs also indicates benthopelagic exploitation by some fish species such as bagrids *Auchenoglanis biscutatus, Chrysichthys furcatus* and *C. nigrodigitatus,* cichlids *Hemichromis bimaculatus, H. fasciatus, Tilapia melanopleura* and *T. zillii*; channid *Channa obscura*; clariid *Clarias gariepinus,* notopterids *Papyrocranus afer* and *Xenomystus nigri*; and phractolaemid *Phractolaemus ansorgii*. Fish scale, showing minimal (0.88%) occurrence, should have been picked up by *T. macrocephala*.

The food and feeding reports here are in some cases consistent and comparable with results from other water systems. One of the specialized feeders encountered in this study, *Channa obscura*, consumed mostly fish (40%). The stomachs contained whole fry of *Hemichromis bimaculatus* and *Mormyrops* sp. of early stage of digestion. The young of this species also fed mainly on subdueable size of insects (20%), shrimps (20%) and little detritus (20%). These observations conform to studies elsewhere of Tetsola (1988) and Odum (1992) that adult *C.obscura* was a piscivorous predator. *A. biscutatus* contained much insects (100%) and shrimps (100%). Odum (1992) also classified it as a predator, but in Lake Kainji, Imevbore and Okpo (1975) categorized *C. obscura* as an omnivore. Insects also occurred most frequently in *Erpetoichthys calabaricus* (60%) and *Xenomystus nigri* (100%), as earlier been found by Tetsola (1988) and Odum (1992). Welcomme (1979) recorded *X. nigri* to be a aufwuchs browser. The cichlid *Hemichromis fasciatus* is more of insectivorous predator (100%) in Jamieson River, the same as the conclusion of Odum (1992) in Ethiope River that it fed frequently on fish (66.67%) and shrimps (66.67%) relates it to the findings of Adebisi (1981) and Tetsola (1988). Tetsola (1988) and Odum (1992), in buttressing up this study, already classified *H. bimaculatus* as an omnivore. It fed on algae (100%), macrophytes (50%), detritus (60%), insects (80%), shrimps (60%) and fish (60%). The bagrid catfish *C. furcatus* was classified as a bottom feeder in River Niger (Imevbore and Bakare, 1970). Umeh (1987) observed it to feed on algae, detritus and insect larvae in River Ase where he described it as a bottom feeder. In this study, it was observed to consume several food organisms probably picked up from Jamieson River bottom and the water column. *C. nigrodigitatus* exhibited a more generalized feeding strategy. However, Tetsola (1988) and Odum (1992) rated it as a planktivore, contrary to Brown (1985) and Umeh (1987) who regarded it as an omnivore. The present study supports the latter two workers just like Welcomme (1979) who classified all generalized predators as omnivores.

Conclusion

The fish fauna is consisted of a multi-species (34.78%) family Cichlidae, a trio (13.04%) family Bagridae, 3 dual species (8.70% each) families, Mochokidae, Mormyridae and Notopteridae, and 6 single – species (4.35% each) families Channidae, Characidae, Clariidae, Phractolaemidae, Polypteridae and Schilbeidae. Food items selected determinatively by fish size and visual cues efficiency, possibly constituted the stomach contents in the Jamieson River fish species. It is seemingly clear that fish exhibited no restriction to a particular depth for feeding purpose because they evidently obtained food items from the water column and the river bottom indicating the omnivorous feeding habit.

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ESTIMATION OF LEAF DAMAGE OF BANANA AFFECTED BY BANANA LEAF AND FRUIT BEETLE, *NODOSTOMA VIRIDIPENNIS* MOST. (COLEOPTERA: EUMOLPIDAE) IN BANGLADESH

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The study was made to evaluate the nature, extent of damage and susceptibility of banana leaf (furled leaf, freshly open leaf and mature leaf) affected by leaf and fruit beetle, *Nodostoma viridipennis* Most. in an orchard in Bangladesh Agricultural University, Mymensingh from October, 1997 to October, 1998. The beetle produced damage symptom with the sign of scars and dried feeding areas on banana leaf. The greatest damage was done to furled leaf bearing 69.8 scars which was 1.3 and 2.0 times higher than freshly open and mature leaf, respectively. The furled leaf being suitable feeding was more susceptible than other categories of leaf and seemed to be the target of colonization of leaf and fruit beetle in the orchard. The growth and development of banana leaf was affected by the damage of *N. viridipennis*. There was significant reduction in size of infested furled leaf. The difference of leaf size in case of freshly open and mature leaf was not so marked.

Key words: Banana leaf, *Nodostoma viridipennis*, Leaf damage.

Introduction

The banana (*Musa* spp.) is a leading tropical fruit in the world market with highly organized and developed industry (Anonymous 1979). As food banana is easily digestible and rich in carbohydrate and minerals (Bhan 1977). One hundred grams of edible portion of banana contain 27.2% carbohydrate, 1.2% protein and 0.8% minerals (Gopalan *et al* 1977). This greatly love, fruit comprises nearly 42% of the total fruit production of this country (Haque 1988) and its financial return is very high (Haque 1983). In Bangladesh 633645 tons of banana was produced from an area of 97935 acres, having an acreage yield of 6.47 tons per acre in 1995-96 (Anonymous 1997). This is in fact very low yield compared with that of other banana growing countries like Argentina (34 t/ha), Costa Rica (33 t/ha), Paraguay (32 t/ha), Senegal (32 t/ha) and Honduras (30 t/ha) (Samson 1986). The yield of banana is affected by so many natural and field factors, banana leaf and fruit beetle pest being major among them.

The banana leaf and fruit beetle have two species namely *Nodostoma subcostatum* Jacoby and *N. viridipennis* Most. The former specie is found in some parts of India and latter is the most destructive pest of banana in Bangladesh (Ahmed 1963). This beetle also attacks on banana in China and India (Hill 1983). Different varieties of banana such as Bihar, Alpan, Champa and Malbhog, are seriously affected by this beetle (Sen and Prasad 1953). Serious damage occurs in the Amrita sagar variety in Bangladesh by banana leaf and fruit beetle (Ahad *et al* 1987). The grub of the beetle feed on roots and the adults feed on the epidermis i.e. the green portion on the ventral and dorsal surface of the leaves and makes irregular patches. The feeding areas dry out, showing spot like structure known as scars and due to this damage photosynthesis is reduced and ultimately growth and yield is affected (Ahmed 1963). The beetle also attacks the fruit causing heavy damage, fruits become blemished and their market value is reduced (Prasad and Singh 1987).

Besides the above works, no further information has been found on *N. viridipennis*. Information on this insect pest is thus scanty in Bangladesh. Therefore, the present study was undertaken to know the nature and extent of damage and susceptibility of banana leaf (furled leaf, freshly open leaf and mature leaf) to *N. viridipennis.*

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Materials and Methods

Experiments were conducted in the banana orchard of the Bangladesh Agricultural University, Mymensingh from October, 1997 to October, 1998. The experiment was followed in a randomized complete block design replicating 3 times having plot size of the orchard 320 m^2 (20m x 16m) and the plants were spaced 2m x 2m. The planting system was hexagonal. Thus, there were 90 plants in this orchard. The commercial variety "Amrita sagar" was grown in the orchard for the present study. Sword suckers of 2-3 kg in weight with 3-4 months of age were used as planting materials. The base of the suckers was cleaned, mostly by pruning the old roots and kept in shade for 2 days for drying up the wound areas. Thus the suckers became ready for planting in the orchard. Selected sword suckers were planted in prepared pits on 23rd October, 1997. Manure and fertilizers were applied according to the recommended doses and methods. Necessary intercultural operations were done at proper time. Irrigation and insecticides were not applied during the study period in the banana orchard. Weekly average temperature, relative humidity and rainfall were recorded from the weather yard located about 1km away from the experimental orchard. The weekly mean temperature ranged from 15.2°C to 30.9°C, relative humidity 67.7% to 93.4% and total rainfalls 0.0 mm to 324.8 mm.

Leaf damage done by *N. viridipennis* was clearly seen as scars on the leaves of growing banana. To measure such damage, three kinds of banana leaves namely, furled, freshly open and mature leaves were chosen to determine their comparative susceptibility. The sampling was made in a complete randomized design with three kinds of leaves as treatments. Three banana leaves as replicates of each kind were used for data collection. Three banana leaves without the insects were included as a control. Each replicate leaf was selected from an individual plant in the orchard.

When the banana leaf began to emerge and remained unopen referred to furled leaf which was caged with nylon net supporting with a bamboo stick free from any pest attack. In this caged furled leaf five orchard collected beetles of *N. viridipennis*, irrespective of sex were released to measure the damage done by them. Freshly open leaf of 8 days old and mature leaf of 16 days old were also caged in the similar manner as mentioned above and five orchard collected beetles were released per leaf. There was control for furled, freshly open and mature leaf without release of insect.

The observation was taken daily in the morning to record the number of scars and damage symptom, produced by the beetles as a result of feeding on epidermis of the leaf. The size i.e. length and breadth of all the caged leaves was measured daily at the time of taking data. Data collection continued up to the mortality of the insects. The data were analyzed statistically and mean differences were adjusted with Duncan's Multiple Range Test.

Results and Discussion

Result of the experiment on the extent of damage determined on the basis of number of scars on the three categories of banana leaves namely furled, freshly open and mature leaves are given in Table 1. The scars were first observed on the furled leaf on July 18, 1998 and the highest mean number of scars was recorded on August 6, 1998. The mean number of scars were 3.2 per furled leaf on the first day and then increased up to 7 days old (fourth week of July) leaf causing 31.5 scars per leaf. The beetles fed less on the same leaf which tended to open and the accumulated number of scars were 36.9-69.8 on the 8 to 21 days old (fourth week of July to first week of August) leaf, when *N. viridipennis* was allowed to feed on the freshly open leaf, the accumulated mean scars ranged 6.7 to 24.2 from 9-12 days old leaf. The feeding damage of *N. viridipennis* was found less on the mature leaf of age 17 to 30 days old compared to furled and freshly open leaf. Scars observed on the mature leaf ranged from 5.3 to 35.9 scars per leaf. The beetle caused more damage to furled leaf than freshly open and mature leaf. It was possible that the beetle could readily feed on the epidermis of the furled leaf having succulent condition compared to mature leaf. These findings also support the results reported by Batra (1952) who observed that *N. viridipennis* would seem to prefer the central rolled up leaf before in unfurled. Sen and Prasad (1953) reported that the central leaves of banana plant forming the top whorl at the crown are worsely affected than the other leaves. Ahmed (1963) also reported that only young leaves are attacked by this kind of beetle.

The loss of growth in banana leaf as measured by the leaf size of banana plants infested by *N. viridipennis* was compared with uninfested leaf on furled, freshly open and mature condition. The data of daily observation are given in Table 2. On the furled leaves, the initial mean length of two days old infested leaf was 11.7 cm and breadth 3.3 cm which was less than that of uninfested leaf 14.3 and 4.1 (length and breadth, respectively). After first week, the size of 9 days old infested leaf was 61.7 cm in length and 31.0 cm in breadth and the size of same age uninfested leaf was 68.7 cm length and 32.0 cm in breadth. The size of both infested and uninfested leaf continued to increase until they became mature. Finally the size of 22 days old infested leaf as 82.7 cm in length and 34.7 cm in breadth and that of uninfested leaf was 91.7 cm in length and 36.7 cm in breadth. Mean leaf size measured after 9 days old infested freshly open leaf was 69.3 cm in length and 32.0 cm in

Table 1

Damage done by *N. viridipennis* (5 beetles) on three categories of banana leaves. Figures represent mean number of scars recorded daily

Sampling date		Mean scars (accumulated number)					
		Furled leaf		Freshly open leaf		Mature leaf	
		Leaf age (days)	Mean scars (no.)	Leaf age (days)	Mean scars (no.)	Leaf age (days)	Mean scars (no.)
July	18	2	3.2	9	6.7	17	5.3
	19	3	7.5	10	12.9	18	10.1
	20	4	13.5	11	18.7	19	14.3
	21	5	19.7	12	24.2	20	18.1
	22	6	25.7	13	29.2	21	21.5
	23	7	31.5	14	33.7	22	24.5
	24	8	36.9	15	37.7	23	27.2
	25	9	41.9	16	41.3	24	29.6
	26	10	46.3	17	44.6	25	31.7
	27	11	50.3	18	47.4	26	33.4
	28	12	53.9	19	49.9	27	34.8
	29	13	57.3	20	52.0	28	35.8
	30	14	60.1	21	53.5	29	35.9
	31	15	62.7	22	54.9	30	35.9
August	1	16	64.9	23	55.5		
	2	17	66.6	24	55.7		
	3	18	67.9	25	55.7		
	4	19	68.9				
	5	20	69.4				
	6	21	69.8				
	7	22	69.8				

Table 2

Size of infested and uninfested furled leaf, freshly open leaf and mature leaf of banana

Sampling		Infested leaf					Freshly open leaf					Mature leaf				
		Leaf	Infested leaf		Uninfested leaf		Leaf	Furled leaf		Uninfested leaf		Leaf	Infested leaf		Uninfested leaf	
date		age (days)	Length (cm)	Breadth (cm)	Length (cm)	Breadth (cm)	age (days)	Length (cm)	Breadth (cm)	Length (cm)	Breadth (cm)	age (days)	Length (cm)	Breadth (cm)	Length (cm)	Breadth (cm)
July	18	2	11.7	3.3	14.3	4.1	9	69.3	32.0	69.7	32.3	17	87.7	33.7	88.0	34.0
	19	3	19.7	4.4	24.0	4.9	10	73.3	32.7	74.3	32.7	18	88.0	34.3	88.7	34.3
	20	4	28.7	6.1	33.3	6.5	11	76.7	33.0	78.3	33.3	19	88.7	34.3	89.0	34.3
	21	5	36.7	7.9	41.7	8.7	12	79.7	33.3	81.3	33.7	20	89.0	34.3	89.7	35.0
	22	6	43.7	10.7	49.0	12.0	13	81.7	33.7	83.0	33.7	21	89.7	34.7	89.7	35.3
	23	7	50.7	14.6	56.7	16.4	14	83.3	34.0	86.0	34.0	22	89.7	35.3	90.3	35.3
	24	8	56.7	19.0	63.0	24.9	15	84.3	34.0	87.7	34.7	23	90.0	35.3	90.7	35.3
	25	9	61.7	31.0	68.7	32.0	16	85.0	34.3	88.7	34.7	24	90.7	35.3	90.7	36.0
	26	10	66.7	31.7	74.0	32.7	17	85.3	34.7	89.0	35.0	25	90.7	35.7	90.7	36.3
	27	11	70.7	32.0	78.3	33.3	18	86.0	35.0	89.7	35.0	26	90.7	36.3	91.7	36.3
	28	12	73.7	32.3	82.0	33.7	19	86.0	35.0	90.0	35.7	27	91.7	36.3	91.7	36.3
	29	13	76.7	32.7	84.7	33.7	20	86.7	35.0	90.3	36.0	28	91.7	36.3	92.0	37.0
	30	14	78.7	33.3	87.0	34.3	21	87.0	35.3	90.7	36.0	29	91.7	36.7	92.3	37.0
	31	15	79.7	33.3	88.7	35.0	22	87.0	36.0	91.0	36.0	30	91.7	36.7	92.3	37.0
August	1	16	80.7	33.3	89.7	35.3	23	87.7	36.0	91.0	36.7					
	2	17	80.7	33.7	90.3	35.7	24	88.0	36.0	91.3	37.0					
	3	18	81.7	34.3	90.7	36.0	25	88.0	36.0	91.3	37.0					
	4	19	81.7	34.3	91.3	36.3										
	5	20	82.0	34.3	91.7	36.3										
	6	21	82.7	34.7	91.7	36.7										
	7	22	82.7 b	34.7 b	91.7 a	36.7 a										

Means of length and breadth respectively of infested and uninfested furled leaf having different letter differed significantly at 1% and 5% level.

breadth and while it was 69.7 cm in length and 32.3 cm in breadth for the uninfested leaf measured after the same time period. After first week, the size of 16 days old infested leaf was 85.0 cm in length and 34.3 cm in breadth and the size of same age uninfested leaf was 88.7 cm in length and 34.7 cm in breadth. Finally, the leaf size recorded 25 days old infested leaf was 88.0 cm in length and 36.0 cm in breadth and that of uninfested leaf was 91.3 cm in length and 37.0 cm in breadth. On the mature leaves, the first data of leaf size of 17 days old infested leaf was 87.7 cm in length and 33.7 cm in breadth and that of uninfested leaf was 88.0 cm in length and 33.7 cm in breadth (Table 2). The final data of mature leaf size of 30 days old infested leaf was 91.7 cm in length and 36.7 cm in breadth and leaf size of 30 days old uninfested leaf was 92.3 cm in length and 37.0 cm in breadth.

The infestation of *N. viridipennis* did not have significant effect on the size (length and breadth) of infested and uninfested furled banana leaf at all ages except 22 days old furled leaf. The final (22 days old) length of furled leaf differed significantly in infested and uninfested condition and the difference of breadth was also significant (Table 2). There were no significant differences in length and breadth of both freshly open and mature infested and uninfested leaves at all corresponding ages (Table 2). Again the infestation of *N. viridipennis* resulted greatly in the reduction of the size of the furled leaf in comparison with the freshly open and mature leaf. Such results were in agreement with the findings of Prasad and Singh (1987) who reported that only young leaves are attacked by the beetle. They also observed that when the young leaves have unfurled into a sort of funnel or are still compactly rolled and older leaves at fully unfurled condition are not found to be attacked.

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PHYTASE PRODUCTION BY FERMENTATION

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To maximize the yield of enzymes by *Aspergillus niger* nutritional and culture conditions were manipulated. The results of the study indicated that 30 –35 percent moist heat sterilized and fortified with 2-5 mg ammonium phosphate per 100 g rice bran in static cultivation, produced maximum amount of phytase in 10-17 days period.

Key words: Phytase, *Aspergillus niger*, Nutritional and culture conditions.

Introduction

After calcium (Ca), phosphorus (P) is the second most important, vital mineral for body structure and functioning of all living cells. It is 25 percent by weight of the total body minerals contents. Eighty percent of the P is bound to the bones of the body and 20 percent in the soft tissues and blood. There it is bound to proteins, lipids, carbohydrates, nucleic acids and vitamins, playing various vital functions such as energy production etc., for all living processes, as a part of many enzymes and co-enzymes.

Deficiency of P shows deprived appetite, stiff joints, muscular weakness, poor fertility and depression of oestrus. For livestock, (milk, meat, eggs and wool production) P deficiency leads to serious problems of low feed intake, stunted growth and poor production in terms of meat, milk and eggs. (Mc Donald *et al* 1981).

Animal sources of P are bone meal, fishmeal and milk etc. These are considered to be good quality sources of P, whereas the plant sources vary from good to poor quality sources for P on the basis of bio-availability of P. The principal storage form of phosphorus in plants, particularly in cereal grains, legumes and plants by-products is phytic acid (Gill 1999) (Fig 1).

Phytic acid shows strong chelating properties as a result of its structure. It forms a variety of complexes with cations, such as calcium, magnesium, copper, zinc, iron and proteins, rendering these nutrients biologically unavailable. The occurrence of protein-phytate complexes reduces the availability of proteins to the poultry because phytate-protein complex, is more resistant to proteolytic digestion than the protein alone (Zyla *et al* 1989).

The interaction of phytic acid with protein, vitamins and several minerals is one of the primary factors limiting the nutritive values of cereal grains and legume seeds (Han and Wilfred 1988). Insoluble Ca^{++} and magnesium salts of phytates occur in cereals and other plant by-products (Bird 1998). Experiments with chicks have shown that P of Ca-phytate is utilized only ten percent as compared to disodium-phosphate. Only ruminants can effectively utilize phytate-P, due to rumen microbial enzymes namely phytases (Mc Donald *et al* 1981) which hydrolyse the plant phytates to inositol and inorganic P which are in turn, hundred percent utilized (Fig 1). Thus hydrolyzing the plant phytates prior to monogastric consumption would increase the availability of inositol and inorganic phosphorus in their diet. Halander *et al* (1996) demonstrated that addition of phytase to poultry feed not only increased the bio-availability and absorption of P but digestibility of dry matter, protein, fiber was also enhanced. Thus many attempts have been made to produce microbial phytase (EC3 1.3.8) by fermentation for hydrolysis of dietary phytates to improve the feed quality (Chang *et al* 1977).

The present studies were initiated for the production of phytases, by *Aspergillus niger* and improving the yield of enzymes by manipulating and optimizing the nutritional and cultural conditions. Various plants and plant by-products were used as fermentation substrates to improve the yield of enzymes. The activity of enzymes so produced was studied on various chemicals and plant phosphorous sources at various pH.

Materials and Methods

Materials. Rice bran, wheat bran, soybean meal, mung beans, and corn meal were purchased locally and used as substrates for fermentation purpose.

Organism and growth conditions for fermentation. Locally isolated strain of *Aspergillus niger* capable of producing phytase enzyme was used for fermentation. Culture was maintained on potato dextrose agar at 30°C. One to two

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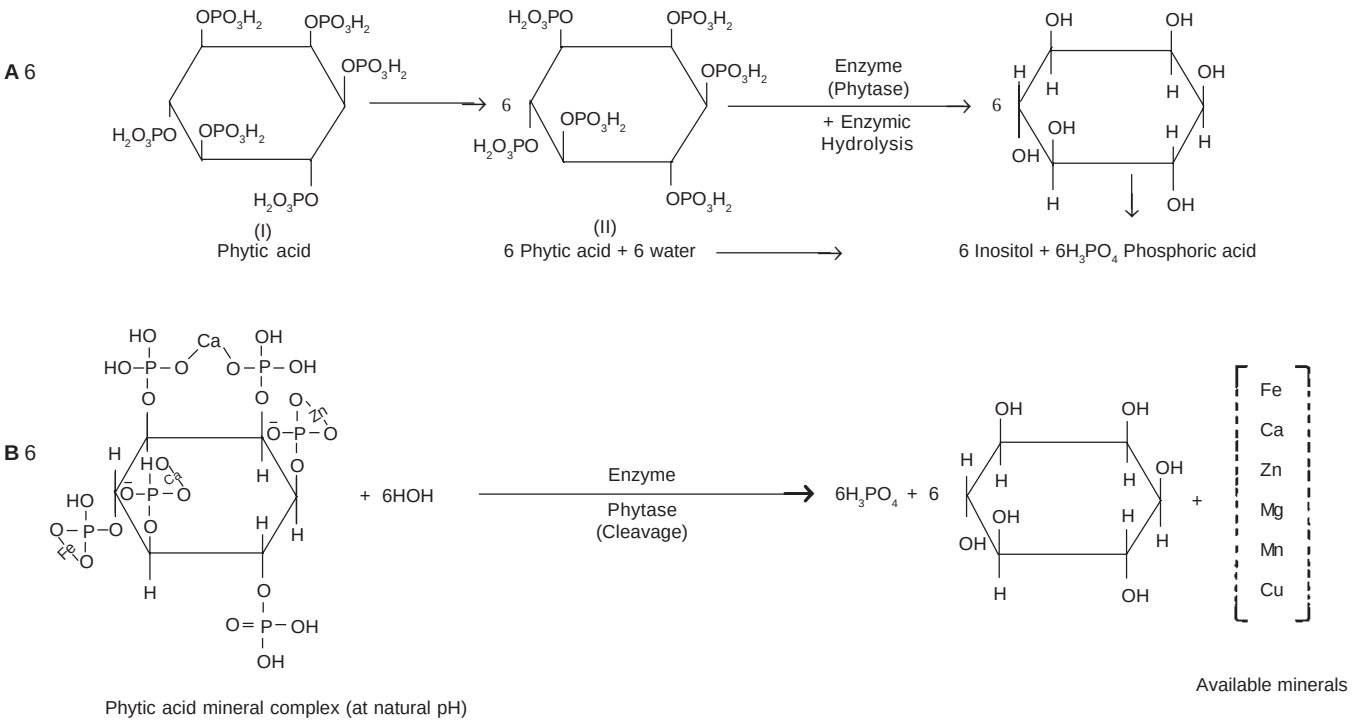

Fig 1. Phytic acid and its products.

week old culture was used for fermentation purpose. Conidia formed on the agar surface were scraped off and collected in normal saline solution. About 10^2 spores/ml were used as an inoculum. The mixtures were steam sterilized before subjecting to fungal growth. In a fermentation run 100g of substrate in 1 litre(L) flasks were inoculated with 1 ml of spore suspension and fermented at 28 to 30˚C for 1-2 weeks as semi-solid stationary culture fermentation.

The following experiments were conducted to standardize the conditions for maximum growth of *A. niger* and enzyme production.

(i) Moisture contents of substrate.

Different levels of moisture from 10 to 90 percent were tested at 28 ± 2˚C. Triplicate flasks containing rice bran as a substrate were inoculated with *A. niger.* The flasks were kept at (28 ± 2˚C) without shaking. Biomass and enzymes production after 10 days were noted.

(ii) Agitated versus static.

The triplicate flasks containing rice bran at 35 percent moisture were inoculated with *A. niger.* The flasks were incubated at 28 ± 2˚C. One set was placed on table and other on shaking machines. After 10 days the quantity of enzymes produced was estimated.

(iii) Effect of added inorganic phosphorous sources.

To the 14 flasks containing 100 g rice bran with (35 percent moisture content) various amounts of inorganic-P (Pi) from 1-1000 mg/flask, were added using ammonium phosphate. Seven flasks were used for static and other seven for agitated growth. *A. niger* was grown for 10 days and the amount of phytase produced was quantified.

(iv) Effect of growth period and enzyme quantity.

The inoculated flasks kept in static fermentation mode were opened after 5 day interval up to 30 days to ascertain the effect of time of fermentation on productivity of enzymes.

(v) Enzyme activity on various purified chemicals of phosphate sources.

Substrate specificity of *A. niger* phosphatases was ascertained using various phosphate sources at two pH values i.e 2.0 and 5.5.

(vi) Effect of various substrate sources.

Defatted rice bran, wheat bran, soybean meal, mung bean and corn meal were inoculated with *A. niger.* The growth of fungus and enzyme activity was studied according to methods of Halander *et al* (1996).

Table 1
Substrate specificity of *A. niger* phosphatase at pH 2.0 & 5.5

Substrate	pH2.0		pH 5.5	
	Pi (μM)[a]	Relative activity[b]*	Pi (μM)[a]	Relative activity*
Na-PNPP	1.058	100[a]	0.792	75 ± 3[a]
Na-Phytate	0.381	36 ± 5[b]	0.514	48 ± 3[b]
Ca Phytate	0.508	48 ± 4[c]	0.485	45 ± 6[b]
$Ca_2P_2O_7$	0.675	63 ± 3[d]	0.162	15 ± 2[c]
$Na_3P_3O_{10}.6H_2O$	0.921	87 ± 8[a]	0.338	44 ± 1[b]
$Na_3P_3O_9$	0.523	49 ± 7[c]	0.092	8 ± 8[c]
$(NH_4)_6\ P_4O_{13}.6H_2O$	0.971	92 ± 4[a]	0.162	15 ± 4[c]
Myoinositol 2-monophosphate	0.276	26 ± 5[b]	0.051	4 ± 2[d]

a. Pi liberated from reaction mixture containing 0.5 ml of substrate (10 μM Pi), 4ml of buffer, and 0.5 ml enzyme during 30 min reaction time.

b. (i) Decimal rounded to whole figure.

(ii) Relative activity on P-nitrophenyl phosphate (PNPP) at pH 2.0 is defined as 100%.

* Same superscript on means in columns show non-significant difference.

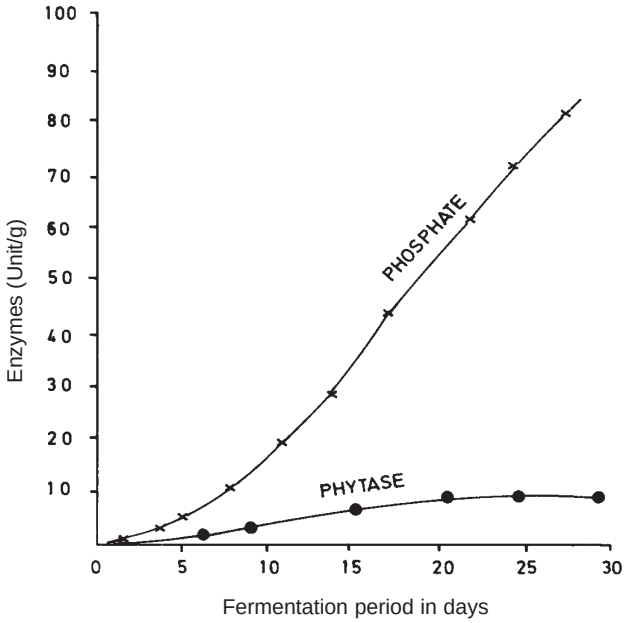

Fig 2. Effect of fermentation period on enzymes production by *A.niger*

A. ANALYTICAL METHODS

i. Determination of phytic acid. Phytic acid in the substrates and samples were determined by a rapid method of Haug and Lantzesch (1983) and inorganic (Pi) as orthophosphorous was determined by the method of Heinonen and Lahti (1981).

Table 2
Effect of inorganic phosphate (Pi) on the production of phytase by *A. niger*

Pi (mg/100 g substrate) (a)	Phytase (Units/g substrate) Static growth (b)*	Agitative growth (c)*
0	8.0 ± 2[a]	4 ± 1[a]
1	8.0 ± 1[a]	4 ± 3[a]
10	82 ± 5[c]	4 ± 1[a]
50	56 ± 3[b]	12 ± 3[b]
100	20 ± 1[a]	10 ± 3[b]
500	12 ± 1[a]	5 ± 2[a]
1000	14 ± 3[a]	4 ± 1[a]

a) Various amounts of ammonium phosphate was added.

b) *A. niger* was grown on semi-solid rice bran for 10 days at 28°C ± 2°C without agitating the substrate on rotating "Wheel shaker".

c) *A. niger* was grown on semi-solid rice bran for 10 days at room temperature with agitation on rotating "Wheel shaker".

*Same superscripts on means in column show non significant differences.

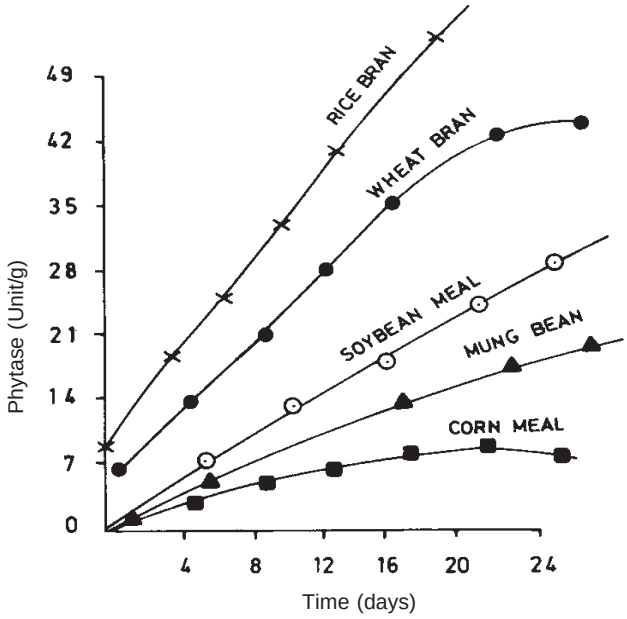

Fig 3. Production of phytase from various substrate sources

ii. Extraction of enzymes and measurement of enzyme activity. Enzymes were extracted by adding ice cold water to the fermented mixture while the medium along with biomass was blended in high speed blender and filtered through a Whatman-4 filter paper. The crude culture filtrate was used as an enzyme source. Phytase activity was assayed by following the release of phosphorous in the form of orthophosphate. The liberated inorganic phosphate (Pi) was determined by the methods of Heinonen and Lahti (1981). In the study where

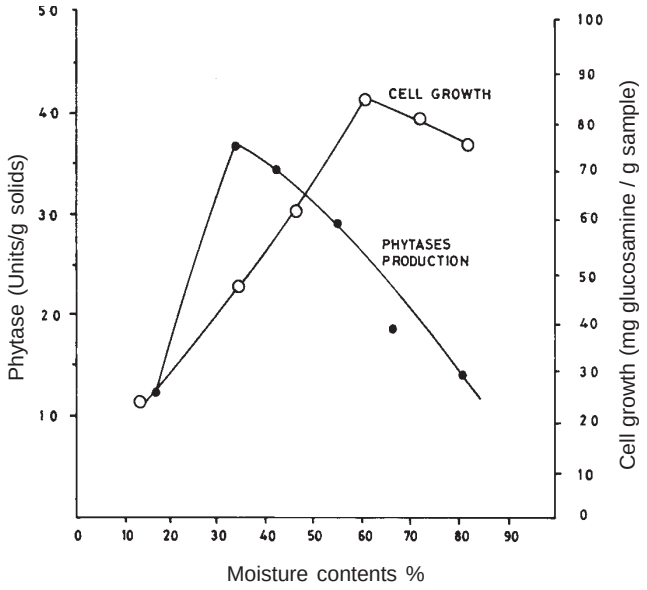

Fig 4. Effect of moisture content on phytase and cell biomass production.

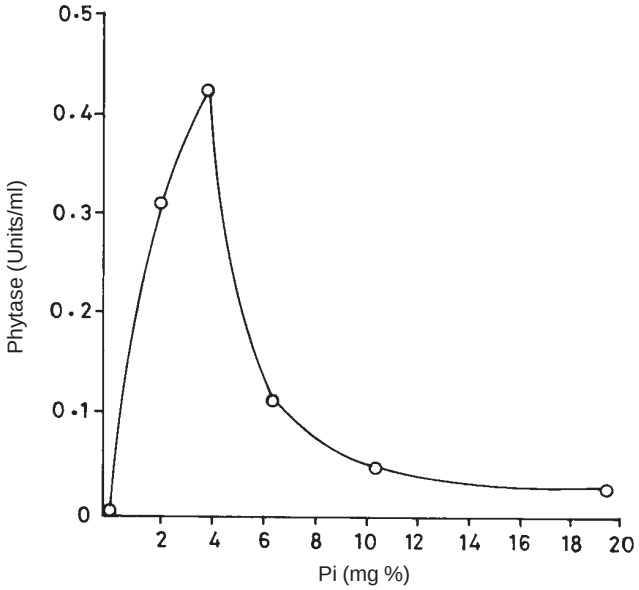

Fig 5. Effect of different levels of Pi addition on phytase production by *A. niger*.

high initial Pi interfered with the determination of Pi liberated by the enzyme, p-nitrophenyl phosphate (PNPP) was used as a substrate and the enzymic activity was reported as phosphatase activity (AOAC 1984). The enzyme reaction mixture contained 0.1 ml of suitably diluted culture filtrate, 3.0 ml of 0.1 M acetate buffer (pH 5.4), and 0.5 ml of 15 µM of PNPP or Na-phytate. The reaction mixture was incubated for 30 min at 37˚C. At the end of the reaction, the colour developed was measured by reading the optical density at 420 nm. Optical density was correlated to the units of enzyme. One unit of enzyme was defined as the amount of enzyme required to liberate 1 mM of Pi per minute under the assay conditions.

Although phytase is a kind of phosphatase, the two enzymes were distinguished on the basis of their activity towards substrates, P-nitro phenyl phosphate (PNPP) for phosphates and sodium phytate for phytase, respectively.

B. ESTIMATION OF GLUCOSAMINE

Glucosamine was determined on the basis of amine-N (AOAC 1984) by the method of Lowry *et al* 1951, using bovine serum albumin as standard. The following buffer systems were used throughout the studies. (pH 1.0 - 2.2) glycerin – HCl (pH 2.8 – 3.2) and citric acid - Sodium citrate (pH 3.0 – 6.2).

C. STATISTICAL METHODS

The data on various parameters were tabulated and subjected to statistical analysis according to Steel and Torrie (1966). The comparison of means was done by Duncan's multiple range test (Duncan 1955) at 5% level of significance, whenever significant difference in means was observed.

Results and Discussion

A. niger produced a non-specific acid phosphatase, rather than phytin-specific phosphatase, which hydrolysed a variety of phosphates (Table 1). The crude enzymes hydrolyzed a variety of phosphates.

The organism produced phosphatases and phytases slowly and the enzyme activity increased continually during the one month period (Fig. 2) but phytase production remained stable after 17 days of growth. However, the levels of enzyme activities towards PNPP (phosphatase) and Na-phytate (phytase) were different depending on the pH. The optimum pH for phosphatase and phytase was 2.5 and 5.5, respectively.

Aspergillus niger grew well on steamed brans i.e. defatted rice bran, wheat bran, than on soybean meal, mung bean and corn meal. The highest amount of phytase was produced on rice bran followed by wheat bran, soybean meal, mung bean and corn meal (Fig 3). The enzyme production was less on corn meal and mung beans as compared to rice bran, wheat bran and soybean. The water content of substrate plays an important role for both cell growth and enzyme production in the solid state fermentation as reported by Cannel and Young (1980) and Toyama (1976).

Addition of water causes swelling of the substrate and facilitates its utilization by the microorganisms. However, the optimal amount of water required varied (Fig 4). About 50 to 60 percent moisture showed the best results in terms of cell growth while optimal moisture content for phytase production was between 30 and 35 percent. The activity of phytase produced was drastically reduced when water contents ex-

ceeded 40 percent. The results are in line with those of Han *et al* (1987) and *Kim et al* (1985), who reported that the optimum water content for cellulase production was lower and the range was narrower than that for cell growth in case of *Tricoderma reseii* and *Sporotrichum cellulophillum* grown on semi solid wheat bran medium.

The initial phosphates in the growth medium at pH 2.0 and pH 5.5 level showed importance for the production of phosphatases (Table 1) *A. niger* required less than 4 mg/100 ml of inorganic (Pi) phosphorous. Ammonium phosphate and sodium phosphate gave the best results. In general, a high level of inorganic phosphate (Pi), inhibits the synthesis of phosphate. The type and amount of phosphatase synthesized are dependent on the concentration of Pi, present in the growth medium (Ohta *et al* 1968; Shieh *et al* 1969).

The addition of more than 5 mg Pi per 100 g substrate (Rice bran) severely depressed phytase synthesis in static growth media (Fig.5).

The mode of cultivation in semi-solid fermentation also affected the optimal concentration of Pi. The static culture produced more enzymes than the agitated culture. Shaking the medium inhibited the production of enzymes. The results are in line with Han and Gallagher (1987) and Tambiev *et al* (1997) who reported product production of extracellular enzymes by *A. niger* when grown on semi solid substrates. The enzymes phytase produced by *A.niger* were efficient to hydrolyse plant phytates. Hence, it can be concluded from the results that 30-35 percent moist, heat sterilized rice bran, fortified with 2-4 mg ammonium phosphate per 100 g rice bran, in static cultivation produced maximum amount of enzyme (phytase) by *Aspergillus niger*, in 10 to 17 days old culture.

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DETERMINATION OF PROTEIN, NITRITE AND NITRATE IN ANIMAL PROTEIN SOURCES IN NIGERIA

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The protein, nitrite and nitrate levels were determined in the body parts of twelve different types of fish, snake, prawn, amphibian and camel, they all serve as animal protein sources. The parts examined for these parameters were: head, gills, internal organs, muscle, bone and skin. All the samples yielded high levels of protein with values ranging between 21.71 g/100 g– 98.80 g /100 g . The nitrite levels ranged between 4.2 mg /kg – 112.06 mg /kg and the nitrate levels ranged between 168.46mg/kg - 4580.67 mg/kg. Nitrate is sparingly used in Nigeria to cure fish and meat. Hence the results of the nitrite and the nitrate might have been due to their levels in the samples. Most of the coefficient of variation values were low in the various sample body parts showing almost homogenous distribution of protein, nitrite and nitrate levels. While the nitrite levels were within the maximum permitted levels, many of the nitrate levels exceeded the maximum permitted levels.

Key words: Determination of protein, Nitrite, Nitrate, Animal protein.

Introduction

Foods can be preserved by fermentation, refrigeration, heat treatment, moisture removal, irradiation and chemical additives. Chemical preservatives are one group of chemicals that are either added intentionally to foods, or appear in foods as a result of processing or storage. The conditions of preservatives, in accord with good manufacturing practices have been enumerated (Ihekoronye and Ngoddy 1985).

Combinations of the salts of nitrites and nitrates have been used in curing solutions and curing mixtures for meats. Nitrites decompose to nitric acid and forms nitrosylheamoglobin, nitrosylmyoglobin and dinitrosylhaemochrome when it reacts with the haem pigments in meats and thereby forms the stable red and pink colors of cured meats (Fox 1966; Giddings 1977). This activity also prevents iron (Fe^{3+}) from catalysing the oxidation of lipids (Zubillaga *et al* 1984). Nitrites can react with secondary and tertiary amines to form nitrosamines, which are known to be carcinogenic. They are currently added as nitrites and nitrates of sodium or potassium. Recent work has emphasized the inhibitory property of nitrites toward *Clostridium botulinum* in meat products, particularly in bacon and canned or processed hams (Frazier and Westhoff 1978). The inhibitory mechanisms by which nitrite inhibits *C. botulinum* have been enumerated (Benedict 1980). Nitrates have a limited effect on a limited number of organisms and would not be considered a good chemical preservative.

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Smoking is a very ancient preservation technique. The smoking of foods usually have two main purposes, adding desired flavors and preservation. The smoking helps preservation by impregnating the food near the surface with chemical preservatives from the smoke or by combined action of the heat and preservatives and by the drying effect, especially at the surface. Intensive smoking does prolong shelf life but it has detrimental effects (Bender 1992).

In view of the biological significance of nitrosamine poisoning it seems desirable to evaluate the extent of contamination of some Nigerian animal protein sources by nitrosamine precursors; the conditions under which these various substances are formed in the food materials and the roles which various factors, which are known to enhance the presence of nitrosamines and their precursors, play in the N-nitrosation reaction *in situ* and under various processing conditions. Inhibition of conversion of nitrites to nitrosamines is also discussed.

Materials and Methods

Samples were purchased at Oba market (Ado-Ekiti, Ekiti State, Nigeria). The samples were identified and then grouped under their major headings: Fish (A – F), Camel (G), Snake (H – I), Prawn (J – K) and Amphibian (L). The samples were further separately divided into their various organs giving a total number of forty samples for analysis. The details of these groupings are shown in Tables 1 and 2.

The separated body parts of the major samples were homogenised in Kenwood major blender.They were transferred into plastic containers, labelled and kept in the freezer for pending analysis. All samples were in dry state after being smoke dried (as purchased).

Determination of moisture and protein. Moisture was determined by the method of the Association of Official Analytical Chemists (AOAC 1990). The dry matter values were calculated by taking moisture content from 100% of the corresponding sample. All proteins were determined by the micro-Kjeldahl method as described by Pearson (1976).

Determination of nitrite and nitrate. Nitrite. The nitrite was extracted from the samples by buffer solution (pH 9.8) and alumina cream. To the sample extract was added 1ml each of sulphanilic acid, 1-naphthylamine and sodium acetate, respectively. The mixture was kept in the dark for color development. The Griess pigment formed was quantitatively measured spectrophotometrically at 540nm (Kamm *et al* 1965).

Nitrate. 25ml of sample extract and buffer solution (pH 9.8) were run through the prepared nitrate reduction column of spongy cadmium and determination of nitrite by the formation of a diazo compound as detailed above. All determinations were in duplicate. The difference between the original nitrite and new nitrite of the same sample gave the value of nitrate.

Nitrite levels have been determined both by HPLC spectrophotometrically. Agreement between the two methods was reasonable (Wootton *et al* 1985). The U V spectrophotometer used was Pharmacia (Pharmacia LKB, Biochrom 4060 UV Visible Spectrophotometer).

Statistical analyses. All the moisture and dry matter (DM) values were reported as g/100g, while all the protein values were reported as g /100g on dry matter basis. Both the nitrite and nitrate levels were reported as mg/ kg on dry matter basis. Mean, standard deviation and coefficients of variation were also calculated (Steel and Torrie 1960). Students T-tests were also calculated to determine the level of significance at P = 0.05 level of the parameters in each group.

Results and Discussion

The common, scientific, vernacular and family names of all the major samples for analysis are shown in Table 1. The sample alphabet representative and sample part; moisture, dry matter and protein values are all shown in Table 2.

The moisture content varied between 5.34 – 35.89 g /100g. The low value of moisture would ensure a longer shelf life for most of the samples. The protein content g/100g ranged between 21.71 – 98.80 on dry matter (DM) basis. These two extremes were shared by the organs of *Malapterurus electricus* whose skin was 21.71g /100g, while the internal organs gave the value of 98.80g /100g DM. The protein levels compared favorably with many unconventional sources of protein in Nigeria. The protein levels of three different types of land snails found in Nigeria ranged between 72.20- 88.83g/ 100g DM (Adeyeye 1996) 54.90 in *Zonocerus variegatus* (Olaofe *et al* 1998) 49–61 in locusts and 45g/ 100g in termites (Mayhew and Macmillan 1988). About 25g of meat will

Table 1
Scientific and vernacular names of the animal protein sources

Major food group		Common name	Vernacular name [a] (Y)	Scientific name	Family
Fish	(A)	Grass-eater	Agbodo	*Distichodus rostradus*	Distichodonida (scaly)
	(B)	Tilapia	Epiya	*Oreochromis niloticus*	Cichlidae (scaly)
	(C)	Trunkfish	Osan	*Gymnarchus niloticus*	Gymnarchidae (scaly)
	(D)	Electric cartfish	Ojiji	*Malapterurus electricus*	Malapteruridae (Non-scaly)
	(E)	Mudfish	Abori	*Clarias anguillaris*	Clarlidae (Non-scaly)
	(F)	Sole	Abo	*Cynoglossus senegalensis*	Cynoglossidae
Camel	(G)	The bactrian camel	Rakunmi	*Camelus bactriamus*	Camelidae
Snake	(H)	Diamond snake	Oka	*Python sp*	Boidae
	(I)	Sunbeam snake	Monamona	*Xenopeltis unicolor*	Xenopeltidae
Prawn	(J)	African river prawn	Ede pupa	*Macrobrachium vollenhovenii*	Palaemonidae
	(K)	Caramote prawn	Ede funfun	*Penacus kerathurus*	Penaeidae
Amphibian	(L)	African bullfrog	Konko	*Rana adspersa*	Ranidae

[a]Y, Yoruba

Table 2
Moisture, dry matter and crude protein levels of the samples

S. No	Sample number	Animal body part	Moisture g/100g	Dry matter g/100g	Protein g/100g
1	A_1	Head	6.90	93.09	67.83
2	A_2	Gills	7.51	92.48	49.64
3	A_3	Internal organs	22.63	77.36	33.20
4	A_4	Muscle	7.14	92.85	61.95
5	A_5	Bone	21.00	79.00	35.10
6	A_6	Skin	6.32	93.67	71.88
7	B_1	Head	13.76	86.23	47.40
8	B_2	Muscle	9.97	90.02	68.54
9	B_3	Bone	10.29	89.70	33.19
10	B_4	Skin	12.79	87.20	72.24
11	C_1	Head	7.37	93.62	44.95
12	C_2	Internal organs	10.64	89.35	84.99
13	C_3	Muscle	10.18	89.81	74.50
14	C_4	Bone	16.07	83.92	51.32
15	C_5	Skin	6.03	93.96	77.43
16	D_1	Head	10.88	89.11	36.19
17	D_2	Internal organs	8.04	92.95	98.80
18	D_3	Muscle	9.13	90.86	21.71
19	D_4	Bone	14.53	85.46	96.97
20	D_5	Skin	8.45	91.54	39.94
21	E_1	Head	6.63	93.36	87.03
22	E_2	Muscle	9.60	90.39	71.62
23	E_3	Bone	5.90	94.09	70.85
24	F_1	Head	10.84	89.15	45.88
25	F_2	Internal organs	5.45	94.54	79.92
26	F_3	Internal	7.18	92.81	67.56
27	F_4	Bone	5.34	94.65	35.80
28	F_5	Skin	8.62	91.37	74.19
29	G_1	Muscle	8.79	91.20	86.89
30	H_1	Flesh + bone	10.96	89.03	26.26
31	I_1	Muscle	8.23	91.76	87.82
32	I_2	Bone	5.69	94.30	64.28
33	J_1	Flesh	18.36	81.63	89.75
34	J_2	Exoske-leton	34.11	65.88	78.13
35	K_1	Whole organism	18.89	81.10	78.66
36	L_1	Skin	16.84	83.15	95.63
37	L_2	Muscle	8.53	91.46	92.42
38	L_3	Bone	5.52	94.47	33.80
39	L_4	Head	6.47	93.52	64.87
40	L_5	Internal	35.89	64.11	90.86

a, All determinations were on dry matter basis

Table 3
Nitrite and nitrate levels of the samples

S.No.	Sample	Nitrite[a,b]	Nitrate [a,b]
1	A_1	34.27	2105.60
2	A_2	12.11	1544.98
3	A_3	32.19	1622.41
4	A_4	12.82	1538.07
5	A_5	18.73	1196.46
6	A_6	42.81	3586.60
7	B_1	41.86	1442.54
8	B_2	28.99	1943.90
9	B_3	65.66	2529.65
10	B_4	36.70	2440.37
11	C_1	75.84	2914.98
12	C_2	13.43	2592.05
13	C_3	37.75	1031.18
14	C_4	47.66	1096.40
15	C_5	34.06	4580.67
16	D_1	43.21	2650.10
17	D_2	51.75	2530.29
18	D_3	37.53	2947.28
19	D_4	51.60	2466.53
20	D_5	4.26	2617.54
21	E_1	4.82	2737.25
22	E_2	4.31	2996.02
23	E_3	4.78	3064.62
24	F_1	40.49	2481.10
25	F_2	88.96	2610.43
26	F_3	54.95	2703.37
27	F_4	76.60	1960.38
28	F_5	111.63	3740.83
29	G_1	112.06	3265.13
30	H_1	81.43	2794.00
31	I_1	39.23	300.78
32	I_2	24.28	501.70
33	J_1	19.60	558.62
34	J_2	25.80	675.47
35	K_1	28.24	415.66
36	L_1	43.30	331.93
37	L_2	34.11	508.20
38	L_3	38.11	969.62
39	L_4	26.63	503.74
40	L_5	49.91	168.46

[a]All determinations were on dry matter basis; [b]Unit, mg/kg

supply 45% of a child's daily need for protein. The addition of 100g of meat to the average Zambian diet would increase the protein by 50% (Jensen 1981). The amino acids in the protein complement, the cereal sources of protein by making good their relative deficiency of lysine (Bender 1992).

Information on the nitrate and nitrite contents of Nigerian foodstuffs is very scanty. Fafunso and Maduagwu (1980) showed that the nitrate and nitrite levels in seven common edible Nigerian leafy vegetables on a fresh weight basis, ranged from 48mg/kg – 270mg/kg NO_3^- and from 0.024 – 0.064 mg/kg NO_2^-. Maduagwu (1976) and Bassir and Maduagwu (1978) reported the presence of nitrates and nitrites in some Nigerian indigenous beverages, namely: palm wine, *pito*, *burukutu*, *ogogoro* and *nono*. Their findings showed that these constituents were widely distributed in beverages

Table 4
Various statistical analysis values for the various analytical results

Sample	$\bar{X}$[a]	SD[b]	CV[c]	T[cd]	Tt[e]
A:A_1-A_6 Moisture	11.92	7.65	64.51	32.98*	2.78
Dry matter	88.08	7.69	8.73	0.29	2.78
Protein	53.27	16.61	31.18	0.02	2.78
Nitrite	25.49	12.71	49.86	0.05	2.78
Nitrate	1932.35	861.36	44.58	0.001	2.78
B:B_1-B_4Moisture	11.70	1.86	15.90	12.06*	4.303
Dry matter	88.29	1.86	2.11	0.06	4.303
Protein	55.34	18.38	33.21	0.01	4.303
Nitrite	43.30	15.82	36.54	0.02	4.303
Nitrate	2089.12	502.19	24.04	0.003	4.303
C:C_1-C_5 Moisture	10.06	3.87	38.47	0.76	3.18
Dry matter	90.13	4.07	4.52	0.62	3.18
Protein	66.64	17.46	26.20	0.15	3.18
Nitrite	41.75	22.77	54.54	0.01	3.18
Nitrate	24443.06	1468.09	60.09	0.001	3.18
D:D_1-D_5 Moisture	10.21	2.65	25.95	1.88	3.18
Dry matter	89.98	2.88	3.20	0.84	3.18
protein	58.72	36.40	61.99	0.01	3.18
Nitrite	37.67	19.62	52.08	0.02	3.18
Nitrate	2642.35	185.15	7.01	0.02	3.18
E:E_1-E_3Moisture	7.38	1.96	26.56	-	-f
Dry matter	92.61	1.96	2.12	-	-f
Protein	76.50	9.13	11.93	-	-f
Nitrite	4.64	0.28	6.03	-	-f
Nitrate	2932.63	172.65	5.89	-	-f
F:F_1-F_5 Moisture	7.49	2.31	30.84	2.68	3.18
Dry matter	92.50	2.31	2.50	1.68	3.18
Protein	60.67	18.96	31.25	0.17	3.18
Nitrite	74.53	27.98	37.54	0.003	3.18
Nitrate	2699.22	649.29	24.05	0.002	3.18
G : G_1	-	-	-	-	-g
H : H_1	-	-	-	-	-g
I : I_1-I_2 Moisture	6.98	1.80	25.79	-	-f
Dry matter	93.03	1.80	1.93	-	-f
Protein	76.05	16.65	21.89	-	-f
Nitrite	31.76	10.57	33.28	-	-f
Nitrate	401.24	142.07	35.41	-	-f
J:J_1J_2 Moisture	26.24	11.14	42.45	-	-f
Dry matter	73.76	11.14	15.10	-	-f
Protein	83.94	8.22	9.79	-	-f
Nitrite	22.70	4.38	19.30	-	-f
Nitrate	617.05	82.63	13.39	-	-f
K:K_1	-	-	-	-	-g
L:L_1-L_5 Moisture	14.69	12.69	86.39	0.34	3.18
Dry matter	85.34	12.68	14.86	0.03	3.18
Protein	75.52	26.36	34.90	0.06	3.18
Nitrite	38.41	8.85	23.04	0.50	3.18
Nitrat	496.39	299.47	60.33	0.01	3.18

[a]$\bar{X}$ =Mean; [b]SD = Standard deviation; [c]CV = Coefficient of variation percent; [d]T = T-test calculated value, [e]T = T-test table value; f = Not calculated; g, See Tables 2 and 3 *Significant at P<0.05.

being hawked for sale in various parts of Nigeria. Nitrate concentrations ranged between 6.60 and 91.30 mg/kg while 0.00–2.90 mg/kg nitrite was detected in these drinks. Amoo (1998) reported levels of nitrate in weaning foods to be 27.00–198.00 mg/kg; weaning food components, 83.70–155.70 mg/kg; vegetables, 263.00– 437.40 mg /kg and alcoholic and non-alcoholic beverages, 0.23–12.96 mg/kg; and the nitrite levels was 2.25 – 5.63 mg/kg for weaning food components, 1.80 – 5.85 mg/kg for vegetables and 5.40–7.20 for alcoholic and non-alcoholic beverages. All Nigerian foodstuffs presumably contain nitrates since nitrates are known to be natural constituents of plant and animal materials.

Our nitrate and nitrite levels in the animal protein sources are shown in Table 3. The nitrite levels ranged from 4.26–112.06 mg/kg, while the levels of nitrate ranged from 168.46– 4580.67 mg/kg ; all determinations were on dry matter basis. The values were greater than the literature values (mostly from plant sources) cited above. The skin of *Malapterurus electricus* (D_5) had the lowest nitrite (4.26 mg/kg) but the levels of nitrite in *Clarias anguillaris* organs were also low with values of 4.82 (E_1), 4.31 (E_2) and 4.78 (E_3). The concentration of nitrites in different body organs appeared to be dependent on the type of animal/fish, body organ, mode of preparation and the likely level of smoking before dryness was achieved. Among the samples available for internal organs investigation, three samples (D_2 = 51.75, F_2 = 88.96, L_5 = 49.91mg/kg) or 60% recorded highest levels of nitrite, while A_3 (32.19) and C_2 (13.43) occupied positions three and five respectively in their groups and made up 40% of the five samples. The head region nitrite levels were highest for E_1 (4.82) and C_1 (75.84); second positions for A_1 (34.27) and B_1(41.86); third position for D_1 (43.21) while both F_1 (40.49) and L_4 (26.63) occupied fifth position each in their groups. For the bones, B_3 (65.66) occupied the highest level in its group; second positions were occupied by C_4 (47.66), D_4 (51.60) and E_3 (4.78); third positions were shared by F_4 (76.60) and L_3 (38.11), while A_5 (18.73) occupied position five in its group. For the skin samples or flesh, A_6(42.81), F_5 (111.63), I_1 (39.23) and J_2 (25.80) all occupied first positions in the nitrite levels in their various groups, while L_1 (43.30) and B_4 (36.70) occupied second and third positions respectively in their groups. G_1 (112.06) was the highest of all the nitrite levels. The sample is usually prepared by first parboiling the muscle of camel and then drying before it is sold. Internal organs of fish were hardly consumed in Nigeria but this will depend on the size and the age of the fish concerned. The levels of nitrite in the head of the samples (fish and amphibian) are of major concern because the fish head is now becoming a delicacy (Okoye 1991) in Nigeria. When fishes are smoked their bones become soft thereby making them suitable for consumption, hence high levels of nitrites in the samples are sources of serious concern. *Oreochromis niloticus* has good values of beneficial minerals (Adeyeye *et al* 1996) but the nitrite levels in it might demand a second thought in its consumption. Both tilapia and the prawns (both red and white) have been recommended for the preparation of fortified cornflour feed as infant formulae substitute (Bamiro *et al* 1994) because of their high levels of proteins and beneficial minerals (Adeyeye 2000). However, the levels of nitrites in the prawns were also high.

The nitrate levels were as varied as the nitrites but the pattern of distribution did not follow the trend of the nitrites. In the skin the nitrates distribution was A_6, F_5 and J_2 in position one in their groups, B_4 and I_1 were in second positions, while L_1 was in the fourth position. In the internal organs, samples A_3, C_2 and F_2 were in the third positions, while D_2 and L_5 were in fourth and fifth positions, respectively. For the head E_1 was in first position, A_1, C_1 and D_1 were in the second positions, F_1 and L_4 were in the third positions, while B_1 was in the fourth position in its group. For bones B_3, E_3 and L_3 were in first positions, C_4 was in position four, D_4 and F_4 were in position five while A_5 was in the sixth position in its group for nitrate level. The following samples: *Xenopeltis unicolor, Macrobrachium vollenhovenii, Rana adspersa* and *Penaeus kerathurus* have nitrate levels below 1000 mg/kg but all other sample values were higher than 1000 mg/kg.

The values of nitrite reported by Wootton *et al* (1985) in cured meat products were generally higher than the values under discussion but the values reported for nitrates were generally lower than our values. Maximum levels of nitrites and nitrates in foods are sodium nitrate (500 ppm), sodium nitrite (200ppm) (Christain and Greger 1990), residual nitrite (70ppm) in meat products and fish meat sausage (in Japan) and 120ppm in bacon (in US) (Kada 1974); in New South Wales they are 125ppm (nitrite) and 500ppm (nitrate) respectively as the sodium salts (NSW 1908). Our results should be seen in the context of maximum permitted levels of nitrite and nitrate incured products in other countries of the world. It is obvious that while nitrite levels are satisfactory, many of the nitrate levels exceeded the maximum permitted levels.

In 1981 the Committee on Nitrite and Alternative Curing Agents in Food of the National Academy of Sciences reviewed the scientific literature on the links between nitrites, nitrates, nitrosamines and cancer. They concluded that circumstantial evidence from epidemiological studies has implicated foods containing high levels of nitrate, nitrite and nitrosamines in the development of cancer, particularly of the stomach and oesophagus. The ingestion of large amounts of nitrate is sometimes a health concern for another reason. It can cause a condition called methaemoglobinemia, which involves the

production of abnormal haemoglobin unable to carry the usual amount of oxygen. This condition is most likely to occur in infants, who may become cyanotic (turn blue from lack of oxygen) if they consume food with high levels of nitrate. This effect may be cumulative, since the methaemoglobin is reduced only at a slow rate (Burden 1961).

There is a special delicacy in Nigeria called "pepper soup" prepared mainly from fish or meat, water, salt and pepper. The pungent taste of red pepper is due to capsaicin ($C_{18}H_{27}NO_3$), while the pungent taste of black and white pepper is due to the alkaloid piperine ($C_{17}H_{19}NO_3$). The piperine content of pepper is as high as 5%. Formation of N-nitroso piperidine, a mutagen by the reaction of nitrite with piperine in an acid solution (human stomach is acidic) has already been reported (Rao *et al* 1981). Other identified nitrosating species and their resources include: NOSCN (from saliva) (Boyland and William 1971), C_6H_4OHNO (from smoke which has many phenolic compounds) and NOCl (from gastric juice, curing salts) (Sebranek and Fox Jr 1985). All these might add to the level of nitrites ingested from meat sources.

The conversion of nitrite to nitrosamine is blocked by ascorbic acid. In this way vitamin C tends to inhibit the formation of carcinogen. The presence of α–tocopherol and erythorbic acid are also effective in reducing nitrosamine formation from nitrites. Currently, the food industry in the United States uses smaller amounts of nitrite to cure foods and through the use of antioxidant keeps the formation of nitrosamines at minimal levels (IFT 1987).

The statistical results for all the samples are shown in Table 4. The groups were A(A_1-A_6), B ($B_1 – B_4$), C ($C_1 – C_5$), D ($D_1 – D_5$), E ($E_1 – E_3$), F ($F_1 – F_5$), G (G_1), H (H_1), I ($I_1 – I_2$), J ($J_1 – J_2$), K (K_1) and L ($L_1 – L_5$). The highest CV values were observed for moisture in $A_1 – A_6$, $E_1 – E_3$, $J_1 – J_2$ and $L_1 – L_5$. The moisture level ranged between 15.90 – 86.39%. The moisture content values were significantly different in $A_1 – A_6$ and $B_1 – B_4$. The CV values was high in protein for $D_1 – D_5$ (61.90) but less than 50.00 in the other sample groups. Nitrite CV levels were greater than 50.00 in $C_1 – C_5$ and $D_1 – D_5$ but less than 50.0 in the rest of the samples. CV for the nitrate levels were high in $C_1 – C_5$ (60.09) and $L_1 – L_5$ (60.33) but less than 50.00 in the rest. The low levels of CV in many of the sample groups was an indication of almost homogenous distribution of the parameters under discussion.

The control of ingoing nitrite levels, use of reductants (ascorbate or erythorbate) and adherence to good processing practices will substantially reduce the problem of nitrosamine formation from nitrites (Dudley 1979). Based on established facts, we are suggesting, that consumption of fruits high in vitamin C should be encouraged, where as of smoke to dry/preserve meat and fish, and consumption of pepper soup should be lessened and great caution should be taken in consuming smoke dried bush meat.

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GENOTYPE X ENVIRONMENT INTERACTION IN RELATION TO DIALLEL CROSSES FOR FLOWER CHARACTERS IN BEAN (*LABLAB PURPUREUS*)

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Diallel analysis includes combining ability effects and components of genetic variation. They were estimated over two environments for flower characters from a half-diallel cross by involving six parents. Both general combining ability (gca) and specific combining ability (sca) were important for all characters with preponderance of additive gene actions. Environment had significant effect on all characters except flowers/inflorescence. Best general combiners were DS52, DS106, DS112 etc. Complete dominance was observed for flowering date in environment-2, flowers/inflorescence in environment-1 and pods/inflorescence in both environments. Over dominance was found for flowering date in environment-1 and partial dominance for flowers/inflorescence in environment-2 and for inflorescence/plant in both environments. Characters were governed by asymmetrical distribution of positive and negative alleles. Dominant and recessive genes were equally distributed for flowering date and inflorescence/plant whereas unequally distributed for flowers/inflorescence and pods/inflorescence. One or two gene groups were involved in flower characters of lablab bean.

Key words: Diallel analysis, Environmental heterogeneity, *Lablab purpureus*.

Introduction

Lablab bean is the most popular winter vegetable in Bangladesh. It is a multipurpose, leguminous crop adapted to dry areas (Kasno and Utomo 1991). Generally its yield, varies greatly with wide seasonal fluctuations mainly due to poor adaptation of cultivars. Genotype-environment interaction is an intrusive factor that plant breeders are to deal with, in developing a high potential variety for wide cultivation. Diallel analysis provides an effective means of obtaining a rapid picture of a genetic control of a character in a number of homozygous lines. It could successfully reveal the major features of a genetic system and predict the out come of the selection in early generations (Yates 1947; Hayman 1954; Griffing 1956; Jone's 1965). In a breeding programme, selection at the early stages is often restricted to a single environment, employing advanced agricultural practices. Selection for increased yield may produce lines adapted to these conditions but lacking the stability, required for commercial cultivation. For example, reselection in the parents of the diallel cross exhibited yield increase in singular experimental traits, but failed to maintain this in wide ranging traits covering diverse environments (Arnold *et al* 1970). G x E interaction have assumed greater importance in plant breeding, that they violently interfere with precise estimates of genetic parameters and with stability of genotype values under diverse environments. The objective of this study was to obtain information on the genetic architecture of the flower characters in two cultural environments and their interaction with environment and also to identify best parents and specific crosses.

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Materials and Methods

Six diverse genotypes of lablab bean (eg. KBS-1, DS-52, DS-106, DS-112, DS-161 and DS-167) were crossed in all possible combinations without reciprocals. Six parents and their 15 F_2s were grown on two cultured environments [eg. Environment-1 (E_1)-early sowing i.e. 20 August, 1996 with fertilizer @ 20g triple super phosphate, 20g muriate of potash and 5g Gypsum per pit and Environment-2 (E_2)-late, sowing i.e. 2 September, 1996 without fertilizer in the pit] in order to study environmental interaction during 1996-97 at the experimental farm of Genetics and Plant Breeding Department, Bangladesh Agricultural University, Mymensingh. A RCB design with five blocks, each representing replication was used for either of environments. Pits were prepared at a spacing of 2m x 2m, between and within the rows. Three to four seeds for each diallel family, including parents, were sown per pit. Necessary cultural operations were done as and when required. Data were recorded from all experimental plants on four characters i.e. flowering date, flowers/inflorescence, inflorescence/plant and pods/inflorescence. Diallel analysis was done following Griffing's (1956) method 2 model 1(fixed effect model). In model 1, the experimental material is regarded as the population about which inferences are to be made. The analysis of variance for combining ability was carried out by using block mean of each entry (diallel family) for individual environment as follows:

$$gca = 1/(p+2)\sum_{I}(Yi+Yii)^2 - 4/pY^2$$

$$sca = \Sigma\Sigma Y^2ij - 1/(p+2)\Sigma(Yi+Yij)^2 + 2/(p+1)(p+2)Y^2..$$
$$i\ \ j$$

Error = SSe

where:

gca = general combining ability
sca = specific combining ability
p = number of parents
Yi = array total of the ith parent
Yii = mean value of the ith parent
Y.. = grand total of the 1/2p(p-1) crosses and parental values
Yij = progeny mean values in the diallel table
SSe = sum of square due to error (obtain from preliminary anova after dividing by the number of blocks)

The gca and sca effect of each character was calculated as follows:

$$gi = 1/(p+2)\Sigma(Yi.+Yii) - 2/pY..$$
$$sij = Yij - 1/(p+2)(Yi+Yii+Y.j+Yij) + 2/(p+1)(p+2)Y..$$

where:

gi = general combining ability effect
sij = specific combining ability effect

The genetic components of variation in F_2 population were calculated according to Jinks (1956) and heritability was estimated as out lined by Verhalen and Murray (1969) for F_2 population.

Results and Discussion

Mean values concerning flowering date and inflorescence/plant suggested that genotypes on an average, performed better in environment-1 than in environment-2. The environmental effects on those traits were highly significant. In environment-1, flowering date was significant and required 14 days more to come to flower than in environment-2, except DS-52. It was probably because most of the bean genotypes in the study were timely fixed and photoperiod sensitive (with exception of KBS-1 and DS-167). Such flowering behaviour with differential sowing dates early with environment-1 and late with environment-2 could be anticipated. Flowering date ranged from 63.4 to 98.4 in E_1 and 47.4 to 105.0 in E_2 among the parental genotypes and from 64.0 to 99.8 in E_1 and 48.0 to 94.8 in E_2 in cross combination, Table 1. Result on the specific performance of genotypes in each environment showed lowest days for flowering, which was in the KBS 1, in environment-2. Whereas Begum and Newaz (2000) reported the

Table 1
Mean values of flower characters in *Lablab purpureus* bean conducted in two environments

Genotype	Flowering date		Flowers/inflorescence		Inflorescence/plant		Pods/plant	
	E_1	E_2	E_1	E_2	E_1	E_2	E_1	E_2
KBS-1	63.4	47.4	8.9	7.6	52.2	17.6	5.2	4.1
DS-52	90.0	87.2	9.9	7.8	70.4	21.0	5.8	4.0
DS-106	98.4	83.8	13.3	12.8	44.4	39.2	9.6	7.9
DS-112	88.8	105.0	14.9	14.7	63.4	34.2	10.0	11.0
DS-161	87.8	73.4	10.8	10.6	48.8	33.8	6.6	7.1
DS-167	63.4	48.0	5.8	8.1	10.4	4.4	3.0	4.6
KBS-1 X DS-52	99.8	80.4	10.1	10.1	75.2	28.2	5.6	6.4
KBS-1 X DS-106	97.4	75.2	11.7	10.6	74.6	40.6	7.4	6.7
KBS-1 X DS-112	92.4	77.6	10.2	10.6	60.0	33.4	6.4	6.8
KBS-1 X DS-161	64.0	48.0	8.5	10.1	49.4	46.8	4.9	5.9
KBS-1 X DS-167	70.6	54.4	9.4	9.1	32.4	24.6	5.2	5.8
DS-52 X DS-106	92.8	82.2	10.3	10.6	61.4	28.4	5.2	7.3
DS-52 X DS-112	96.0	87.6	11.7	11.7	78.2	39.0	8.1	6.6
DS-52 X DS-161	99.4	75.8	10.3	9.6	57.2	41.4	7.1	5.5
DS-52 X DS-167	96.4	83.0	11.1	10.7	59.0	29.2	7.3	7.3
DS-106 X DS-112	88.4	77.4	10.3	11.1	51.0	38.6	6.7	8.0
DS-106 X DS-161	84.2	73.2	11.2	11.4	58.0	58.2	7.9	8.2
DS-106 X DS-167	87.2	71.4	10.5	11.3	34.6	24.0	6.0	8.3
DS-112 X DS-161	88.4	73.6	9.3	10.6	50.0	34.2	5.2	7.1
DS-112 X DS-167	90.6	94.8	8.8	9.1	46.4	30.0	5.7	6.0
DS-161 X DS-167	87.0	73.6	10.0	8.5	46.0	27.4	6.2	4.2
SE(±)	2.7		0.74		9.02		0.67	

lowest days for flowering in DS-167. Late sowing and absence of added fertilizer (environment-2), drastically reduced the number of inflorescence/plant. The lesser number of inflorescence meant lesser productive pods and finally lower yield. The analysis of variance for combining ability (Table 2) showed that both gca and sca variance were significant in both environments for flowering date, flowers/ inflorescence and pods/ inflorescence; indicating that additive as well as non-additive gene action were important for these characters. In inflorescence/plant, additive gene action was important for both the environments. Singh and Singh (1981) reported the predominant role of additive genetic variance for flowering date and non additive component to be more important for pods/inflorescence. Singh *et al* (1986) and Hossain (1993) reported additive and non-additive component to be important for flowers/ inflorescence, whereas Sharma and Pandey (1996) in Urd bean (*Vigna mungo*) reported inflorescence/plant to be principally affected by additive gene action. The gca x env. was highly significant for flowering date and inflorescence/plant, revealed the influence of environment on genetic parameters. Non significant gca x env. and significant sca x env. for pods/inflorescence, indicated that the non-additive effects were more influenced by environment than the additive effects, controlling this traits (Khanam *et al* 2000). Keeping in view, the result obtained in two environments for flowers/inflorescence, no significant interaction of heterogeneity between combining ability was observed. Most of the genotype for all the characters are selected on the basis of significant value except flowering date. It is selected by negative value because it indicates the general capacity of early parent to transmit its behaviour to progenies in cross combinations with other parents. Analysis of gca effects (Table 3) showed that KBS 1 and DS-167 emerged as the best general combiners for early flowering in both environments and DS-52 and DS-112 were the best combiners of lateness. Occasionally, late flowering and late fruiting into the season may also be considered desired characteristics, though not as much as earliness. These genotype may be used in breeding for early or late flowering behaviour. The estimation of sca effect in environment-1 (Table 3) revealed that KBS-1 x DS-161 that was the best specific cross, combining good and moderate parents followed by DS-106 X DS-112, in which cross combination of both parents were poor combiner for earliness. These two best specific crosses for earliness exchanged their position in environment-2. The latter cross was the best in environment-2, while DS-106 and DS-112 were by far the two best general combiners for flowers/ inflorescence and pods/inflorescence for environment-1 and environment-2, respectively. For both the characters, the highest sca effect was recorded from the cross DS-52 x DS-167, in both the environmental conditions. Interestingly, both of the parents in this cross were poor general combiner for flowers/ inflorescence. In pods/ inflorescence out of 15 crosses, 5 crosses KBS-1 x DS-161, DS-52 x DS-167, DS-106 x DS-112, DS-112 x DS-161 and DS-161 x DS-167 showed highest performance for pods/inflorescence. These crosses could be utilized for exploiting non-additive gene action for pod characters in further breeding programme. In case of inflorescence/ plant the best parents were DS-52 in environment-1 and DS-161 in environment-2, followed by DS-106 in both the environments. As regard sca effect, KBS-1x DS-106 and DS-106 x

Table 2

ANOVA (ms) for combining ability in two environments and their interaction for flower character in bean

Item	df	Flowering date	Flowers/inflorescence	Inflorescence/plant	Pods/plant
Environment 1					
gca	5	277.640***	6.86***	747.97***	5.17***
sca	15	89.950***	2.14***	82.27	1.73***
Error	80	10.560	0.44	109.20	0.30
Environment 2					
gca	5	647.410***	7.91***	296.94***	6.69***
sca	15	84.320***	1.20***	67.43	1.45***
Error	80	4.060	0.67	53.57	0.60
Item x *env. interaction*					
gca x env.	5	91.030***	0.68	307.63**	0.58
sca x env.	15	21.670***	0.56	37.58	1.03
pooled error	160	7.314	0.55	81.38	0.45

*,p<0.05; **,p<0.01; ***,p<0.001

Table 3
General and specific combining ability effects on flower character in *Lablab purpureus* bean

Genotype	Parameter	General combining ability		Specific combining ability											
				KBS-1		DS-52		DS-106		DS-112		DS-161		DS-167	
		E_1	E_2	E_1	E_2	E_1	E_2	E_1	E_2	E_1	E_2	E_1	E_2	E_1	E_2
KBS-1	Flowering	- 7.23***	- 11.74***	-	-	-	-	-	-	-	-	-	-	-	-
DS-52	date	6.95***	7.38***	- 0.05	0.25	-	-	-	-	-	-	-	-	-	-
DS-106		4.75***	2.83***	1.27***	0.45	- 0.34	- 0.09	-	-	-	-	-	-	-	-
DS-112		3.07***	12.08***	0.86*	1.34***	0.54	0.36	- 0.62	0.17	-	-	-	-	-	-
DS-161		- 1.27	- 4.17***	- 0.80*	0.54	- 0.21	- 0.23	1.54***	1.49***	- 1.07**	- 0.77*	-	-	-	-
DS-167		- 6.27***	- 6.39***	- 0.30	0.20	- 0.03	0.78*	- 0.28	0.29	- 0.18	0.03	0.57*	- 0.47	-	-
KBS-1	Flowers/	- 0.58***	- 0.88***	-	-	-	-	-	-	-	-	-	-	-	-
DS-52	inflorescence	0.13	- 0.52*	0.21	1.01	-	-	-	-	-	-	-	-	-	-
DS-106		1.03***	1.11***	0.88	0.03	- 1.23*	- 0.35	-	-	-	-	-	-	-	-
DS-112		0.98***	1.34***	- 0.48	- 0.25	0.23	0.57	- 2.08***	- 1.00	-	-	-	-	-	-
DS-161		- 0.18	- 0.12	- 1.04*	0.70	0.23	- 0.06	0.07	0.07	- 1.84***	- 0.95	-	-	-	-
DS-167		- 1.37***	- 0.93***	1.01*	0.53	2.06***	1.85**	0.53	0.76	- 1.17*	- 1.62**	1.19*	- 0.81	-	-
KBS-1	Inflorescence/	2.72	- 1.99	-	-	-	-	-	-	-	-	-	-	-	-
DS-52	plant	12.14***	- 2.07	6.85	0.15	-	-	-	-	-	-	-	-	-	-
DS-106		- 0.73	5.43*	19.12*	5.05	3.70	- 7.07	-	-	-	-	-	-	-	-
DS-112		4.79	2.36	- 1.00	0.93	7.77	6.60	- 6.15	- 1.30	-	-	-	-	-	-
DS-161		- 2.03	6.36**	- 4.78	10.33	- 6.40	5.00	7.27	14.30**	- 6.25	- 6.62	-	-	-	-
DS-167		- 16.88***	- 10.09***	- 6.73	4.58	10.25	9.25	- 1.28	- 3.45	4.5	5.63	11.42	- 0.97	-	-
KBS-1	Pods/	- 0.65***	- 0.81**	-	-	-	-	-	-	-	-	-	-	-	-
DS-52	inflorescence	- 0.03	- 0.65*	- 0.20	1.22*	-	-	-	-	-	-	-	-	-	-
DS-106		0.91***	1.02**	0.68	- 0.06	- 2.9	0.34	-	-	-	-	-	-	-	-
DS-112		0.90***	1.27***	- 0.27	- 0.27	0.77	- 0.63	- 1.53***	- 0.89	-	-	-	-	-	-
DS-161		- 0.05	- 0.16	- 0.84*	0.23	0.79	- 0.35	0.59	0.71	- 2.04***	- 0.62	-	-	-	-
DS-167		- 1.083***	- 0.66**	0.47	0.67	1.94***	2.05***	- 0.26	1.33*	- 0.53	- 1.22*	0.92*	- 1.56**	-	-

*,p<0.05; **,p<0.01; ***,p<0.001

DS-161 were the best cross combinations in both environments. The parent combination of these crosses was average x poor and good x good combiners. The importance of DS-106 and DS-161, was already indicated as good general combiner for inflorescence/plant as well. D measures only additive effect, H_1 and H_2 measure only dominance effect. Excess dominant allele was present in the parent of flowering date, flowers/inflorescence and pods/inflorescence (Table 4), because their F value was positively significant and excess recessive allele was present in inflorescence/plant. For all the characters, differences between parents and crosses were present which is measured by h^2 value. Degree of dominance was measured by $[(H_1/D)/4]^{1/2}$ parameter. Complete dominance was observed in flowering date for environment-2, flowers/ inflorescence for environment-1 and pods/ inflorescence for both environments due to the $[(H_1/D)/4]^{1/2}$ value which was equal to unity. Partial dominance was found for flowers/ inflorescence in environment-2 and for inflorescence/plant in both environments and over dominance was reported for flowering date in environment-1 and Khanam (1999) also reported the similar result. All traits were governed by asymmetrical distribution of positive and negative alleles as $H_2/4H_1$ values were smaller than 0.25. The dominant and recessive genes were equally distributed in flowering date for both environments and inflorescence/plant in environment-1because $[(4DH_1)^{1/2}+F]/[4DH_1)^{1/2}-F)]$ value was greater than one. Those genes were unequally distributed in flowers/ inflorescences and pods/ inflorescences for both environments and inflorescence/plant in environment-2 due to $[(4DH_1)^{1/2}+F]/[4DH_1)^{1/2}-F)]$ value which was less than unity. One or two gene groups were involved in all traits which are governed by h^2/H_2. High heritability was found in flowering date, flowers/ inflorescence and pods/inflorescences. Mode-rate heritability was observed in inflorescence/plant. Narrow sense heritability of 1.2 in both environments for flowers/inflorescences, must however, be treated as "Spurious" for heritability estimate cannot exceed 1. Singh and Singh (1981) and Reddy (1982) also reported high heritability for different cha-racters in *Lablab purpureus*. The characters that are gover-ned by the genetic system, predominantly of additive nature can be selected in early generation and those characters largely controlled by dominance or non-additive effects required to be selected in later generations. In the present study, number of inflorescence/plant was controlled by additive gene effects mostly and flowering date by non-additive effects largely, as suggested by the results obtained in both environments. For other character the genetic architecture appeared to have been influenced by the changes in environment.

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Table 4

Estimates of some genetic components for flower characters in bean conducted in two environments

Genetical parameter	Flowering date		Flowers/inflorescence		Inflorescence/plant		Pods/inflorescence	
	E_1	E_2	E_1	E_2	E_1	E_2	E_1	E_2
D	209.11	518.99	10.16**	8.08***	328.66***	117.96***	6.92***	6.94***
F	300.92*	686.18***	24.28***	14.44***	- 80.54	- 49.57	15.04***	13.47**
H_1	1309.28***	1497.22***	34.86***	2.16*	- 620.64**	5.18	26.82***	17.69
H_2	1058.09***	1072.13***	19.66***	5.71	- 299.63	136.38	18.68***	9.84
h^2	21848***	15659.85***	284.15***	290.87***	7127.0***	2675.6***	107.19***	115.6***
E	11.84	4.03	0.43	0.68	108.03	53.79	0.29	0.61
$[(H_1/D)/4]^{1/2}$	1.25	0.85	0.93	0.61	- 0.69	0.105	0.98	0.80
$\frac{H_2}{4H_1}$	0.20	0.18	0.14	0.12	0.12	6.58	0.17	0.14
$\frac{(4DH_1)^{1/2}/4+F/2}{(4DH_1)^{1/2}/4-F/2}$	3.71	8.03	- 7.89	- 5.37	1.43	- 0.34	- 20.19	- 10.29
$\frac{h^2}{H_2}$	20.65	14.61	14.45	50.95	- 23.79	19.62	5.74	11.76
h^2n (narrow sense heritability)	0.48	0.92	1.20	1.20	0.51	0.33	0.95	0.98
h^2b (broad sense heritability)	0.89	0.97	0.80	0.59	0.33	0.40	0.84	0.65

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ELEMENTAL ANALYSIS OF *CALENDULA OFFICINALIS* PLANT AND ITS PROBABLE THERAPEUTIC ROLE IN HEALTH

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Elemental analysis of crude ethanolic and aqueous extracts of *Calendula officinalis* plant and its individual parts i.e. flowers, leaves and roots reveal the presence of major, minor and trace elements in appreciable quantities. Elements detected in crude ethanolic and aqueous extracts of whole *Calendula* plant are calcium 5319, 2464 ppm, sodium 16990, 14745 ppm, potassium 79782, 71617 ppm, magnesium 6059, 9060 ppm, iron 74.459, 22.94 ppm, copper 8.05, 1.27 ppm, manganese 37.22, 36.96 ppm, zinc 51.50, 34.00 ppm, nickle 5.10, 2.45 ppm, cobalt 1.39, 0.98 ppm and cadmium 0.40, 0.50 ppm. While chromium and lead were found to be present in 1.01 ppm in ethanolic and in traces in aqueous extracts.

Highest percentages of iron 125, 28.16 and copper 80.59, 10.19 ppm were found to be present in ethanolic and aqueous extracts of root portion respectively. The highest quantity of nickle 8.46 ppm was found in aqueous extracts of roots while in ethanolic extract it was 7.10 ppm in flowers. *Calendula officinalis* and its individual parts were found to be rich in calcium, sodium, potassium and magnesium contents which are not only essential but are also required for the haemostatic control of the body.

Key words: Herbal plant, Elemental analysis, *Calendula* plant.

Introduction

Calendula officinalis Linn, Compositeae, commonly known as Genda, Zargul or Marigold (Kirtikar and Basu 1933; Chopra *et al* 1985) is not only a spring board for monastries and garden but has an array of uses in drugs, foods, feed, beverages, dyes, culinary, cosmetics, perfumery industries and at ceremonial religious occasion. (Kirtikar and Basu 1933; Robert 1968; Chopra *et al* 1985; Mamchur *et al* 1987; Loggia *et al* 1994; Kalvatchev *et al* 1997; Vasudevan *et al* 1997).

Medicinal uses of *Calendula* plant and contemporary pharmacological studies have proved that the effectiveness of *Calendula* plant mostly depends upon its organic constituents (Mamchur *et al* 1987; Jakupovic *et al* 1988; Loggia *et al* 1994; Vasudevan *et al* 1997) but the effectiveness of in organic constituents of *Calendula* plant has been neglected despite of the fact that many states of health impairments and disease are linked to elemental inbalance (Under-Wood 1977; Shahnaz *et al* 1994; Liu Longhim and Cushiu 1997). Efforts have been made to develop health protective products based on trace elements and traditional plants (Wang Chang Chung and Waugh 1997). The survey of literature on *Calendula* plant reveal that it is an effective remedy against epileptic fits, fever, pain, kidney trouble, muscular pain, hypertension, skin diseases, cuts, wounds, ulcers, leucorrhoea, cancer and bleeding piles. The plant is also effective as hypocholesterolemic, antimicrobial anti-inflammatory, haemostatic and against urinary tract infection (Kirtikar and Basu 1933; Chopra *et al* 1985; Mamchur *et al* 1987; Krazhan and Garazha 1999; Klossner and Axid 2000).

Present work, therefore, is an attempt to provide information on elemental contents of *Calendula officinalis* plant in order to have an unbiased scientific look along with the realization of munificent role of elements present in the plants as remedial agents. These elements could act as an adjuvant or as an alternate effective therapy of controlling, combating or treating certain diseases/disorders along with the organic constituent in the plants.

Materials and Methods

Experimental procedure. Collection of plants: The *Calendula officinalis* plant was collected from the floral beds of PCSIR Laboratories Complex, Karachi in the month of April, washed throughly and dried in air before use.

Estimation of moisture. Samples (2.0g), each of whole *Calendula* plant, flowers and leaves, were taken in a weighed china dish. It was dried in an oven at 130°C ± 1°C till the weights were constant. The loss in weight was taken as moisture content. Estimation was carried out by the method of (AOAC 1984).

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Estimation of ash. Weighed materials, each of dried whole *Calendula* plant, flowers and leaves were ignited in a muffle furnace at 550°C, until whitish grey ash was obtained. The resultant masses were cooled and treated with 1.0ml concentrated nitric acid and reignited for 1.5h to render them completely free from carbon. Samples were cooled, reweighed and percentage ash content of each samples was calculated.

Extraction. Fully grown mature plants were taken, washed and dried in air. Whole plant and its individual parts i.e. flowers and leaves 1.0 kg each and roots 0.25 kg were chopped into small bits and soaked in 95% ethyl alcohol for 96 h with continuous agitation for 10 h day. The solvent was then decanted and concentrated *in vacuo*. The resultant gel like mass was called as ethanolic extract. A part of the ethanolic extract was then partitioned with water and petroleum ether (2:1, v/v). Aqueous layer was then separated and concentrated under reduced pressure at room temperature into semi-solid mass to obtain the aqueous extract.

Reagents. All reagents, used in present study, were of Analar grade; triple glass distilled water was used in the preparation of all solutions and standards.

Digestion of samples. Test materials (1.0g) each of alcoholic and aqueous extract of whole *Calendula* plant and their individual parts (i.e. flowers, leaves and roots) were digested with concentrated nitric acid (HNO_3), in acid washed pyrex tubes at 120°C, till the solution was clear and the volume was reduced to about 1.0ml and then was made up to 10.0 ml with distilled water. A sample blank was also prepared similarly. Estimations were made using standard addition technique (Price 1978). The dilutions were made such as to keep the concentration of different elements within the linear range of absorbance.

Estimation of elements. Sodium and potassium estimations were made using Corning Flame Photometer, Model No.410. HITACHI Z-8000 Atomic Spectrophotometer with Zeeman background corrector and microprocessor control was used in the flame version for other elemental analyses using standard addition technique (Price 1978).

Results and Discussion

Moisture contents of whole *Calendula* plant, leaves and flowers were 81.83, 85.39 and 85.94% while ash contents on dry basis were 11.62, 14.78 and 7.21% respectively. The reported values are average of replicates.

Elemental analysis of the ethanolic and aqueous extracts of whole *Calendula* plant and its individual parts, i.e. flowers, leaves and roots reveal the presence of major, minor and trace elements in appreciable quantities (Tables 1 and 2). Thirteen elements i.e. Na, K, Ca, Mg, Fe, Zn, Mn Cu, Ni, Co, Pb, Cr and Cd were found to be present in ethanolic extract of whole *Calendula* plant and leaves. Cobalt and cadmium were not detected in flowers while nickel, cobalt and chromium were not present in root part. Lead and cadmium were found to be present in traces in the alcoholic extract of the root part. On the other hand, all the thirteen elements were found to be present in the aqueous extract of whole *Calendula* plant and leaves. Chromium and lead were detected in traces in whole plant. While chromium was not found in roots. Iron and copper were found to be present in higher concentration in root portion of both ethanolic and aqueous extract (Tables 1 and 2) as compared to other elements while nickel was high only in the aqueous extract of the roots. High percentage of sodium, and potassium were found to be present in root part while calcium and magnesium in leaf portions of both ethanolic and aqueous extract.

Data obtained on the elemental analysis of both ethanolic and aqueous extracts of whole *Calendula* plant and its individual parts lie in conformity with the available elemental data obtained on the basis of their percent concentration of total human body weigh which indicate Ca as major with average concentration of 1% or more, Na, K and Mg as minor from 1 to 0.01% and Fe, Zn, Cu, Cd, Pb, Mn, Co, Ni, and Cr below 0.01% as trace elements, (Table 3) (Heydron 1987).

Furthermore, all these detected elements have a well-documented record of their therapeutic, preventive, medicinal and biological role in many pathophysiological conditions and functions through various mechanisms (Under Wood 1977; Liu-Longhim and Cuishu 1997) Moreover, Ca, Mg, Na, K and Fe and taken specially as essential part of die (Chattergee and Rana 1997).

All the trace elements detected in ethanolic and aqueous extract of the whole *Calendula* plant and its individual parts confirm their uses in different ailments.

Calcium, sodium, potassium and magnesium are nor only the chief electrolytes but are also essential for the haemostatic control of the body. They are essential for the nervous system, maintenance of fluid volume in the body, contractile mechanism of muscles, maintenance of correct rhythm of heartbeat, clotting of blood etc. (Fleck 1971; William 1971; Shahnaz *et al* 1994; Edward *et al* 1995; David 1996; Liu-Longhim and Cushiu 1997). Calcium is also a constituent of liposusin and age pigment (Fleck 1971; William 1971). Calcium and magnesium deficiencies result in disease of respiratory system (Hou-Xiaolin *et al* 1995).

Chromium, Co, Zn, Mg, and Cu play an important role in controlling elevated insulin level, impaired glucose tolerance and

Table 1
Elemental analysis of *Calendula* plant (alcoholic extract)

S.No.	Name of elements	Whole plant (ppm)	S.D.±	Flowers (ppm)	S.D. ±	Leaves (ppm)	S.D. ±	Roots (ppm)	S.D.±
1.	Ca	5319.00	18.19	886.00	15.00	1930.00	17.32	284.50	3.24
2.	Na	16990.00	13.58	3447.00	16.70	5409.00	15.62	42764.00	26.48
3.	K	79782.00	19.15	14772.00	18.02	27775.00	19.22	49344.00	25.55
4.	Mg	6059.00	9.94	468.00	6.55	2631.00	4.40	2467.00	6.92
5.	Fe	74.45	3.93	72.30	0.53	39.50	0.25	125.00	2.25
6.	Cu	8.05	0.70	7.49	0.05	5.50	0.09	80.59	0.25
7.	Mn	37.22	0.52	3.26	0.01	119.50	0.74	8.22	0.04
8.	Zn	51.50	2.62	5.15	0.02	54.50	0.52	34.54	0.34
9.	Ni	5.10	0.10	7.10	0.08	4.00	0.01	-	-
10.	Co	1.39	0.06	-	-	2.00	-	-	-
11.	Cr	1.01	-	0.23	-	0.65	-	-	-
12.	Pb	1.01	-	0.77	-	3.00	0.01	Traces	-
13.	Cd	0.40	-	-	-	0.40	-	Traces	-

S.D. = Standard Deviation.

Table 2
Elemental analysis of *Calendula* plant (aqueous extract)

S.No.	Name of elements	Whole plant (ppm)	S.D.±	Flowers (ppm)	S.D. ±	Leaves (ppm)	S.D. ±	Roots (ppm)	S.D.±
1.	Ca	2464.00	17.350	5892.00	26.22	6982.00	59.778	195.22	5.570
2.	Na	14745.00	29.460	8793.00	29.086	20030.00	98.173	34549.00	65.390
3.	K	71617.00	20.290	40889.00	69.555	95797.00	98.658	101919.00	71.330
4.	Mg	9060.00	26.162	1759.00	15.710	6532.00	38.736	1002.00	18.350
5.	Fe	22.94	1.048	13.13	0.205	11.00	0.467	28.16	0.030
6.	Cu	1.27	0.059	0.77	0.039	2.00	0.176	10.19	0.070
7.	Mn	36.96	0.210	29.13	0.726	163.00	2.810	8.64	0.040
8.	Zn	34.00	0.075	34.00	0.262	29.50	0.700	50.10	0.220
9.	Ni	2.45	0.027	3.17	0.153	6.50	0.050	8.46	0.045
10.	Co	0.98	0.025	-	-	2.56	0.010	-	-
11.	Cr	Traces	-	0.08	0.030	0.10	-	-	-
12.	Pb	Traces	-	0.77	0.036	5.50	0.050	4.66	0.026
13.	Cd	0.50	0.036	0.05	0.011	0.45	-	1.21	0.036

elevated serum cholesterol concentration, a risk factor for cardio vascular disease (Mertz 1969; Kelevey 1975; Under-Wood 1977). Clinical studies made on preparations rich in Cr, Co and Zn have proved their therapeutic role in depigmentation hypothermia, hypotonia, central nervous system degeneration, apnea, blood vessel rupture, hypocholesteremia, anaemia and diarrhea (Kelevey 1975). Nickle is also an important trace element. It plays an important role in structure of membranes, lipid metabolism, regulation of RNA and DNA of some enzyme systems. Furthermore, its deficiency can effect iron absorption (Mertz 1974; Vohora 1983). Magnesium inhibits calcium phosphate precipitation in tissues and thus protects against nephrocalcinosios and formation of renal stones (Cramer 1932). Lower physiological doses of Cr appears to decrease serum cholesterol, atherosclerosis, aortic plaque and increases glucose uptake (Kelevey 1975). Cobalt is an essential co-factor for vitamin-B_{12}, while Fe, Cu, and Co are required for the production of red blood cells and prevention of pernicious anaemia. Most research studies indicate the iron deficiency leads not only to behavioral changes but also to biochemical chages in brain. Iron also plays a pivotal role in erythropoiesis and in many intracellular reactions of oxygen transport (Edward 1995 and David Chappal 1996).

Table 3
Elements in reference of man/human body

S.No	Elements	Symbol	Percent of total body wt.	Overall body content mg/kg
1.	Calcium	Ca	1.40%	-
2.	Potassium	K	0.20%	-
3.	Sodium	Na	0.14%	-
4.	Magnesium	Mg	0.03%	-
5.	Iron	Fe	-	10-100
6.	Zinc	Zn	-	10-100
7.	Copper	Cu	-	1-10
8.	Lead	Pb	-	1-10
9.	Cadmium	Cd	-	1-10
10.	Manganese	Mn	-	0.1-1
11.	Cobalt	Co	-	0.01-0.1
12.	Chromium	Cr	-	0.01-0.1
13.	Nickle	Ni	-	0.01-0.1

A perusal of literature on elemental composition of herbal drugs indicates that Ca, Cu, Fe, K, Mg, Cr and Zn have important therapeutic role in diabetes mellitus, specially Cr, Mg, Mn and Zn. Manganese and Zinc are very effective for the treatment of male sterility with low sperm count (Han and Mingsong 1997). The metallic compounds are also valued for their cosmetic and skin therapeutic effects (Berne *et al* 1985; Dumas and Bonte 2000). British National Formulary listed 11 elements for dermatological use. Among the known essential elements, only Zn seems to have received sufficient attention. It is used in powder, paste, ointments, creams, shampoos, lotions and antidandruff preparations. Zinc is also used as a life saving agent in cases of acrodermatitis enteropathica, acne, alopecia aerata, dissecting cellulitis of scalp, wound, ulcer, eczema and dissecting superficial malignant skin lesions known as Mohs surgery (William 1971; Stromberg and Agren 1984; Klossner and Axel 2000). Furthermore, trace elements like K, Zn, Mg, Fe and Cu are also used in the formulation of antibacterial insecticides along with plant extracts (Yang 2000).

It can be easily concluded that the elements detected in *Calendula* plant have a well-documented record of their curative nature and fully support the curative nature and fully support the curative and therapeutic role claimed to cure many ailment. Efforts are needed to cull out the full therapeutic role of *Calendula officinalis* plant based on its elemental composition for better healing and cure.

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PHOSPHORUS ADSORPTION IN REPRESENTATIVE SOILS OF PESHAWAR VALLEY (NWFP)

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Soil incubation experiment was conducted to study some aspects of phosphorus (P) cycling in soil series of Peshawar Valley. Phosphorus adsorption before and after incubation was studied, physico-chemical properties and AB-DTPA extractable P were determined. Soil samples containing 19 to 22% (w/w) moisture were incubated at 25°C for 7 weeks. The amount of P adsorbed by the soil from KH_2PO_4 solution (0 to 50 µg/ml) before and after incubation was calculated by the difference between the amount of P in solution after 24 h shaking and the initially amount was present. These soils contain $CaCO_3$, which might adsorb organic P, or form insoluble Ca phosphates. The adsorption isotherm plots highlight the ability of these soils for P sorption. Soil having high amount of $CaCO_3$ and clay have more affinity for P adsorption in the soil system.

Key words: Phosphorus, Adsorption, Isotherm, Langmuir, Freundlich.

Introduction

The significant role of phosphorus (P) in sustaining soil fertility has long been recognized. In spite, of the marked advancement in our knowledge about P behaviour in soil, understanding about P dynamics in the soil-water-plant system is still far from completion. The P transformation, fixation and release characteristics in the soil-plant system have been the subject of numerous research investigators, (Ansiaux 1977; Sarir 1989; Iqbal 1990,) but in actual practice, the most pertinent issue is to know how much phosphorus can be made available to the growing crop from the native soil pool.

Soils of Pakistan are deficient in nitrogen and nearly 80% of them lack adequate amount of phosphorus to support prosperous agriculture (Nasir *et al* 1990). Some of these soils are inherently deficient in phosphorus due to peculiar nature of parent material (lacking of P bearing mineral, apatite) while others have been made deficient due to mining of this element through intensive cultivation of high yielding crop varieties with no or little P replenishment in the form of inorganic or organic fertilizers, farm yard manure and crop residues for decades. Still in some soils though P contents are adequate, their specific characteristics such as high pH and $CaCO_3$ content hamper its availability to crop plants. Moreover, in contrast to nitrogen no addition of P through natural biological means takes place. Thus a great majority of soils need extraneous supplementation of P, in order to sustain crops yields.

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In Pakistan, the annual use of phosphatic fertilizer has reached approximately 0.4 million tons, against nitrogen, which is 1 million tons (Iqbal 1990) and 551.1 N tons against 2087.6 N tons (Agriculture Statistic of Pakistan 1998). This indicates that the use of phosphorus in relation to nitrogen in the country is imbalanced with nitrogen phosphorus ratio of 3:1, against the required ratio of 1: 1 or 2:1 for most crops. In addition, the phosphatic fertilizers are highly inefficient and the crop plants can make use of only 15 - 25% of the applied phosphatic fertilizers. A significant part of the phosphatic fertilizer is lost through different mechanisms, of which both chemical and biological transformation into insoluble nonavailable form play an important role. In order to maximize the fertilizer phosphorus use efficiency and to know mechanism responsible for P solubility and insolubility, understanding the different aspects of Phosphorus transformation in the phosphorus cycle is important.

Phosphate sorption capacity is an important soil characteristic that affects the plant response to phosphate fertilizer application. Phosphate sorption isotherms have been used to estimate phosphate requirement and to evaluate the residual effects of phosphate fertilizers (Sarir 1989). The inclusion of this parameter with other soil P test may improve the accuracy of the estimate of P fertilizer requirements for optimum crop yield. In the present study, adsorption maxima and bonding energy constants of some selected soils were determined. Such study will give useful information on the adsorption capacity of these soils and will help in the judicious use of phosphatic fertilizers for growing agricultural crops.

Materials and Methods

The different soil series were identified with the help of Soil Survey Staff (1967). Soil samples of Tarnab, Mardan and Warsak soil series in NWFP were collected up to 20 cm depth for these studies. A composite sample was prepared from five sub-samples taken from the same soil series. The samples were labelled and brought in sealed plastic bags to the laboratory at the earliest and spread out on clean plastic sheets. Any vegetation, roots, stone etc were discarded. The samples were partially air-dired to permit sieving through a 2 mm sieve with minimum disturbance. Precautions were taken to minimize any chemical and biological changes in the samples during the preparation process e.g drying of samples were made at low temperature (possible room temp.) and they were kept away from chemical containing phosphorus. The sieved samples were thoroughly mixed and refrigerated in tied plastic bags until they were to be used for further experimentation.

Soil analysis. The sieved soil samples were analyzed for various physico-chemical properties. Soil texture was determined by hydrometer method. A 1.0 N NaOH solution was used as dispersing agent as described by Moodi *et al* (1959). The pH in 1:5 soil water suspension was determined with the help of pH meter using the method outlined by Richards (1954). Electrical conductivity (EC) in 1:5 soil water suspension was determined with the help of electrical conductivity meter by using the method of Richards (1954). Alkaline earth carbonate was determined by using acid neutralization methods as outlined by Black (1965). Organic matter was determined by the method of Jackson (1958) and moisture was determined by the method of Atikinson *et al* (1958). AB-DTPA extractable P were determined by the method of Soltanpour Schwab (1977).

Incubation procedure. Soil samples were incubated for 7 weeks using the incubation techniques of Jenkinson and Powlson (1976) with some modification. The 120 g soil sample on oven dry basis from each soil were weighed in eighteen plastic vessels 6 cm in diameter, and 10 cm deep. The soil moisture was kept at field capacity leveled by the addition of distilled water. The soil containers were then placed in 1.5 liter kilner jars. The kilner jar lids were then screwed on to make it air tight. The soils were incubated at 25 ˚C for a period of 7 weeks. To keep the moisutre percentage constant, the distilled water was added to the containers at weekly intervals (readjusting any loss in the weight of the soil). Fresh air was also allowed to enter the kilner jar for 10-15 min.

Adsorption procedure. To obtain data for an adsorption isotherm, 5 g oven dry equivalent fresh soil samples were weighed in 250 ml flasks, 50 ml of solution containing 0,5,10,20,30, 40 and 50 µg P/ml were added to these flask. Two to four drops of toluene were added to flasks to stop microbial activities if any in the soil solution. The flasks were then tightly sealed and equilibrated by shaking on a rotating plate form shaker for 24 h at room temperature. Following the equilibration, the solution was centrifuged and filtered. A control set of phosphorus solution having the same concentration (0-50 µg P/ml) was also run, for measuring the initial phosphorus content in the solution. Aliquots of the clear filtrates and control solutions were analysed for phosphorus using spectronic 601 Spectro Photometer by the method of Watanabe and Olsen (1962).

Results and Discussion

Physico-chemical properties. The physico-chemical properties of the soils series used in this study are presented in Table 1. The pH values of all soil series were neutral (pH 7.2). The organic matter content was low and varied between 0.71 to 0.91%. All the soil series analyzed were found to be calcareous, with $CaCO_3$ content ranging from 7.32 to 24.76%. Tarnab soil series contained highest calcium carbonate (25%), while Mardan had the lowest (7.32%). Electrical conductivity of the soils generally ranged from 0.24 to 0.32 dSm^{-1}. The percent clay of the most soil series ranged from 9.20 to 33.20 (clay loam to loam).

Ammonium bicarbonate extractable P (later called, extractable P) ranged from 0.58 to 4.15 mg P/kg soil, in which Tarnab soil series had the lowest P (0.58 mg P/kg soil), while Warsak soil series had the highest extractable P (4.15 mg P/kg soil) Table 1. Because of convenience in use and due to the absence of any better alternative chemical extractants are widely used for testing the available P status or the P supplying power in the soil.

Phosphorus adsorption. The graphical plots of the amount of phosphorus adsorbed by the soil against the equilibrium solution concentrations were also plotted in the form of the Langmuir and Freundlich isotherm equations. Adsorption isotherms, Langmuir and Freundlich plots are illustrated in Fig 1-3, 4-6 and 7-9, respectively. Phosphorus adsorption constants calculated from Langmuir and Freundlich plots are given in Table 2.

Adsorption isotherm illustrated in Fig 1-3, indicate that each soil exhibited different adsorption characteristics. The great variation in P adsorption by different soils have important bearing in soil test crop response correlation studies. It seems that both clay initial P and $CaCO_3$ content play important role in P adsorption by these soils as were seen from adsorption isotherm plots. Phosphorus adsorption was higher in Tarnab soil than in the Mardan and Warsak soils, this probably owed to the presence of more $CaCO_3$ and clay content in the former

Table 1
Physico-chemical properties of soils used

Soil series	AB-DTPA extractable P mg/kg	pH*	EC* dSm^{-1}	O.M	Moisture	$CaCO_3$	Clay contents
				%			
Tarnab	0.58	7.2	0.27	0.71	22.78	24.76	33.20
Mardan	1.18	7.2	0.24	0.91	19.72	7.32	9.20
Warsak	4.15	7.2	0.32	0.91	22.05	11.88	14.40

* Soil pH and EC were determined in 1:5, soil, water extract.

Table 2
Phosphorus adsorption constant for some soil series of NWFP calculated for Langmuir and Freundlich plots

Soil series	Langmuir plot			Freundlich plot		
	b µg/g	K L/mg	R^2	Intercept	Slope	R^2
Tarnab-BI	500	0.200	0.46	1.66	0.85	0.99
Tarnab-PI	500	0.200	0.51	2.06	0.79	0.97
Mardan-BI	-	0.000	0.01	1.08	1.25	0.99
Mardan-PI	1000	0.033	0.16	1.38	1.05	0.99
Warsak-BI	-	0.000	0.00	0.60	1.31	0.96
Warsak-PI	333	0.100	0.68	1.41	0.77	1.00

BI: Before incubation, PI: Post or after incubation.

soil. According to Goswami and Singh (1979), the content of clay explains 77% of the variation in P fixation of the soil and it is desirable to determine the clay content of the soil as a routine test for the purpose of recommending the dose of P fertilizers. Phosphorus adsorption was also substantially affected by incubations, and P adsorption was greater after incubation than before incubation in all soils. However, the effect was greater in Tarnab soil which contains low initial P, as compared to Mardan and Warsak soils, which generally contain more initial P.

Generally, an adsorption isotherm is a curve relating the amount of a substance adsorbed at an interface to its concentration at equilibrium in the medium in contact with the interface. Phosphate retention increased linearly with increasing P concentration or addition. Mean P retention by the soil was 80 ± 3% of added P (Agbenin 1998). However, the adsorption isotherm obtained in Fig. 1-3, continued to increase gradually with increasing P concentration, and a well defined maximum is not obtained. Such observations were reported by Bache and Williams (1971) for soils with a concomitant uniform level of bonding energies for P. The data may, however, be treated according to the number of adsorption equations. The data were fitted to the Langmuir adsorption equation:

$$\frac{C}{X/M} = \frac{1}{Kb} + \frac{C}{b}$$

Where C is the equilibrium concentration of phosphate, X/M is the amount of phosphate adsorbed per unit mass. The b is a phosphate adsorption maximum and K is a term relating to the energy of adsorption.

The relationships between values of C/X/M and C were not liner (Fig. 4-6) and as such do not conform to the conventional Langmuir equation. The constants derived from the Langmuir equation in Table 2, indicated great deviation from the linear form of the equation.

The bonding constant, K L/mg, for P adsorption by these soils, calculated from the slope and intercept ranged from 0.03 to 0.2 L/mg with a mean value of 0.13 L/mg, the average value of bonding constant by Rennie and McKercher (1959) was 0.14 L/mg, for Soils ranging in pH from 6.5 to 7.2, while 0.92 and 4.39 L/mg were reported by Olsen and Watanabe (1957) for alkaline and acidic soil, respectively.

The K value, the energy with which P is released, bears an inverse relationship to the maximum P releasing capacity (b value) of soil (Dev *et al* 1979). The b value of the under study soils ranged from 333 to 1000 µg/g soil Table 2. Different

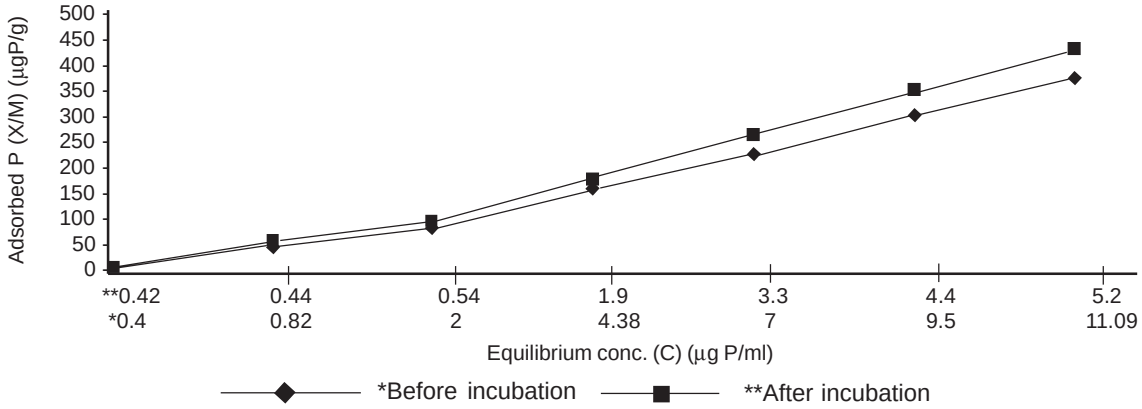

Fig 1. Adsorption isotherm before and after incubation for Tarnab soil series.

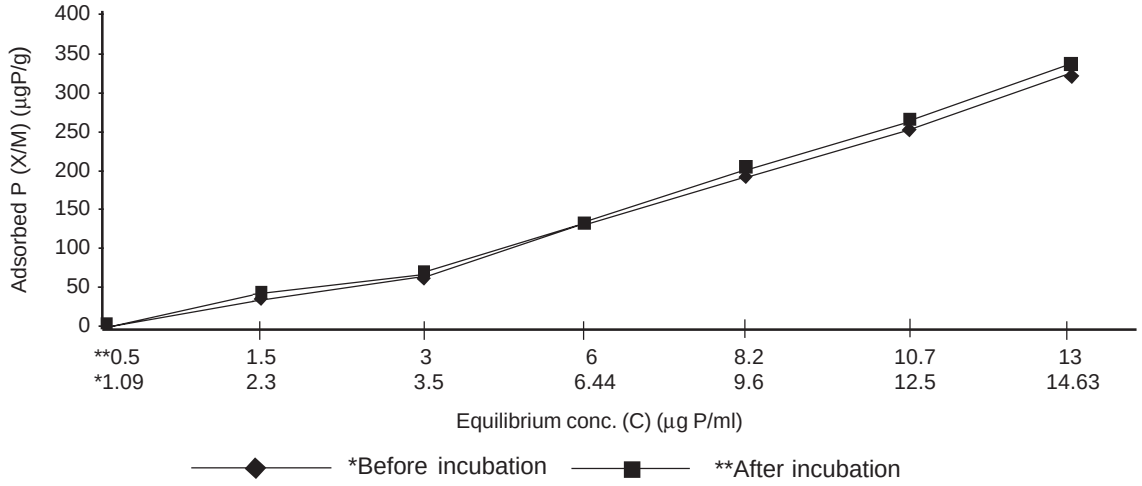

Fig 2. Adsorption isotherm before and after incubation in Mardan soil series.

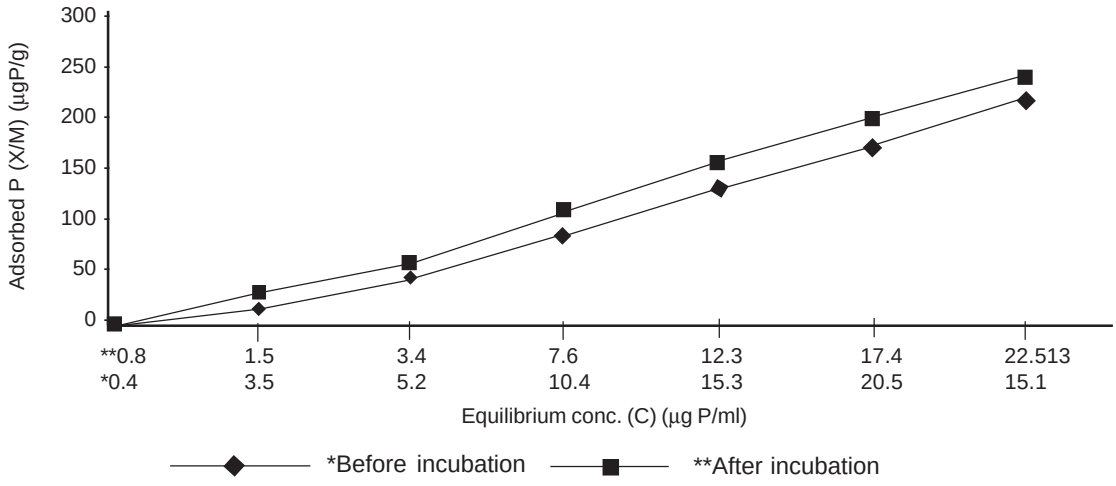

Fig 3. Adsorption isotherm before and after incubation in Warsak soil series.

values of adsorption maximum have been reported by different workers. Sarir (1989) reported as much as 1800 µg/g soil adsorption maximum for Scottish soil, with pH ranged from 3.0 to 7.2. Olsen and Watanabe (1957) found the adsorption maximum ranging from 105 (alkaline) to 300 (acidic) µg/g soil. The maximum phosphorus releasing capacity reported by Dev *et al* (1979) for different Indian soils ranges from 16-900 µg/g. It is assumed that one hec-

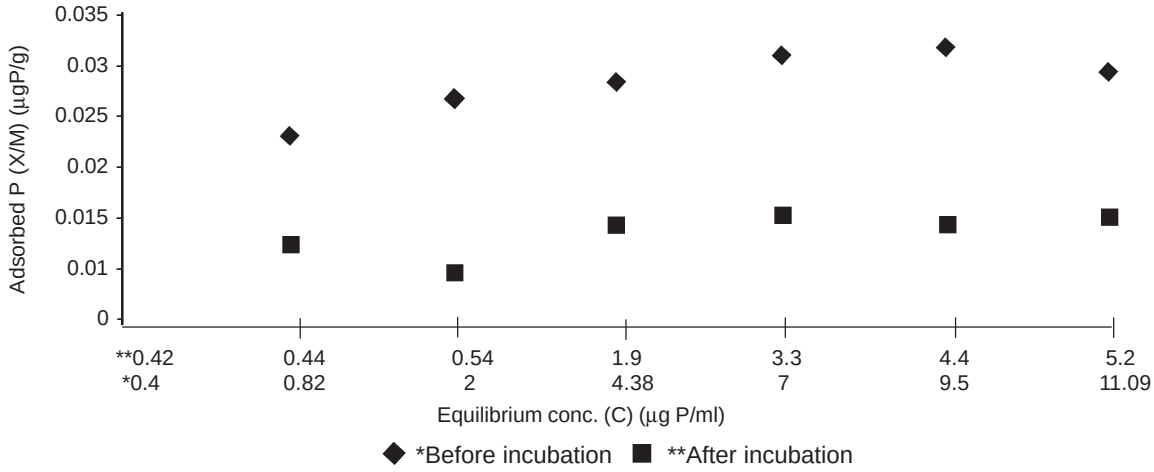

Fig 4. Langmuir adsorption isotherm before and after incubation in Tarnab soil series.

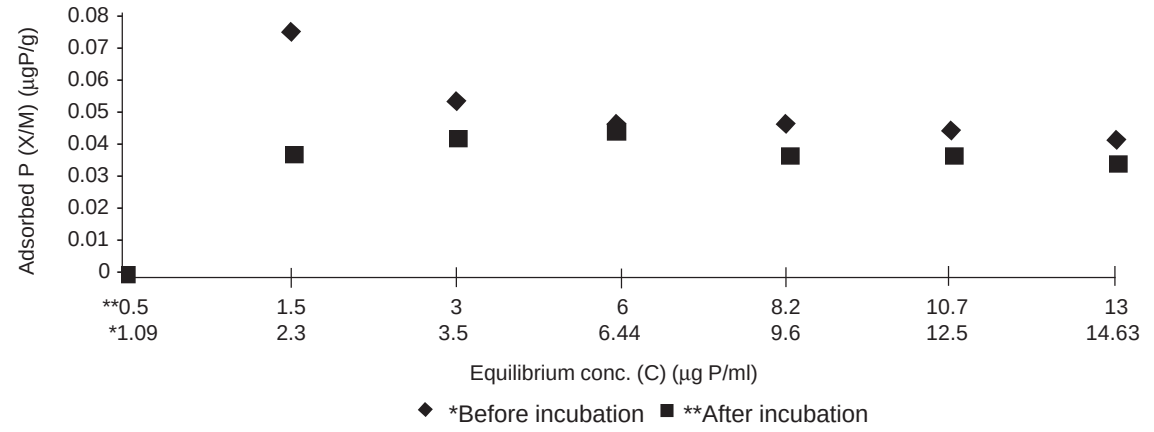

Fig 5. Langmuir adsorption isotherm before and after incubation in Mardan soil series.

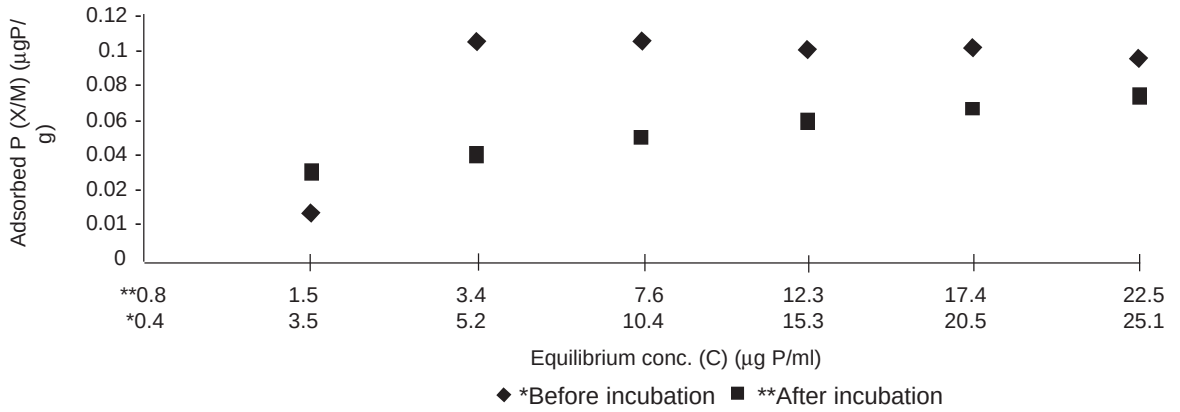

Fig 6. Langmuir adsorption isotherm before and after incubation in Warsak soil series.

tare contain 1800 tons of soil, an equivalent value of adsorption capacity of 0.54 to 1.0 ton P ha^{-1} can be expected in these soils. However, these constants are markedly affected by experimental errors, and little weight can be placed, if the experiments are not correctly tackled (Rennie and McKercher 1959). Therefore, considerable work is needed for selecting the concentration at which to measure the isotherm slope, within convenient experimental

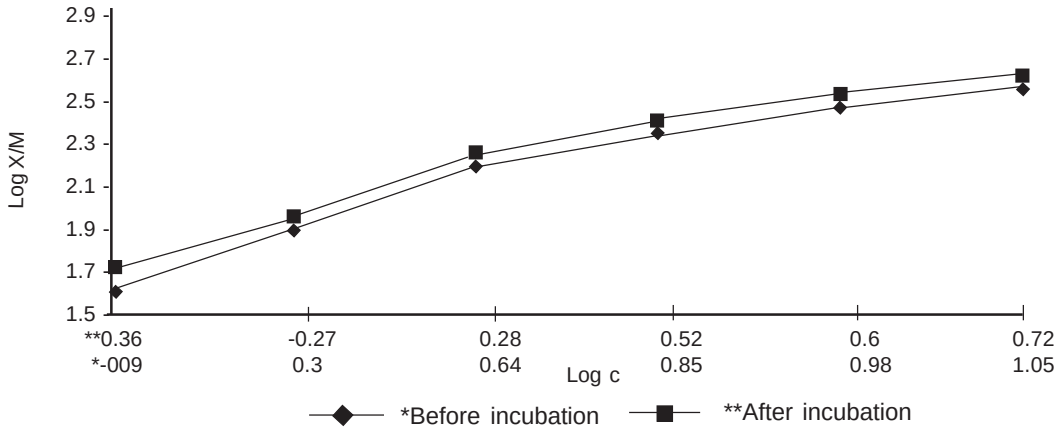

Fig 7. Freundlich adsorption isotherm before and after incubation in Tarnab soil series.

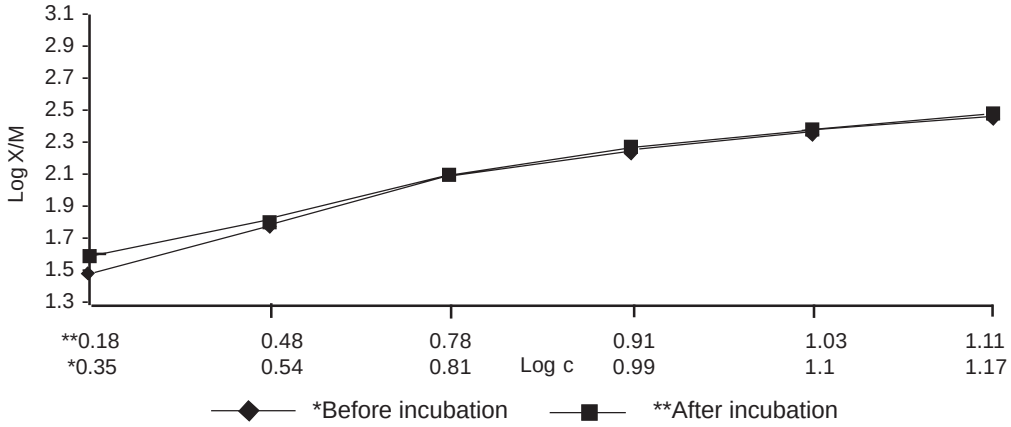

Fig 8. Freundlich adsorption isotherm before and after incubation in Mardan soil series.

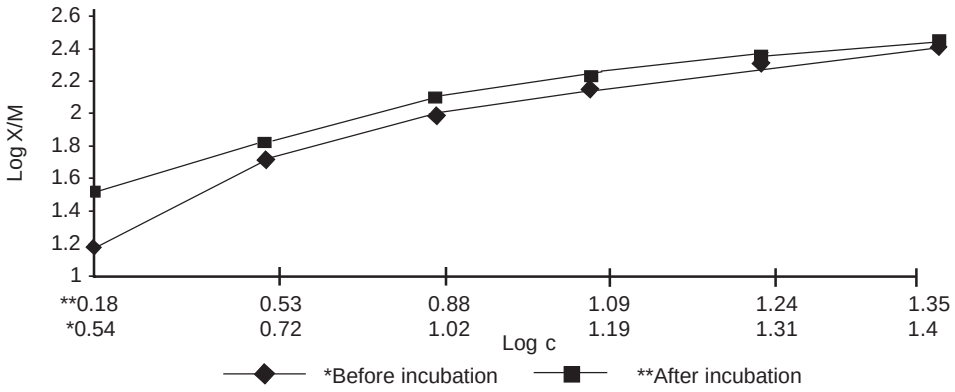

Fig 9. Freundlich adsorption isotherm before and after incubation in Warsak soil series.

range for each soil with different of sorption properties.

If data fail to conform to the Langmuir equation, the less demanding Freundlich equation can often be used successfully (Hinrich *et al* 1985). The linear equation is derived as follow:

$$\text{Log } x/m = 1/n \log c + \log k.$$

Where 1/n is the slope of the regression line, K and n are empirical constant.

Adsorption data were also fitted to the Freundlich adsorption and values log x/m versus log c were plotted. Experimental data proved to obey the Freundlich adsorption isotherm Fig 7-9. Phosphorus adsorption by all three soils (before and after incubation) appeared to conform well to the conventional Freundlich equation and gave a good fit of the linear form of the equation. The fits (R^2) of the straight lines to the Freundlich plot are given in Table 2. The R^2 values indicated a good fit of Freundlich plots.

Castro and Torrent (1998) concluded that the relative significance of Ca phosphate precipitation and P adsorption on variable charge surfaces in calcarious soil varies widely depending on soil properties and amount of P is added.The amount and rate of Ca phosphate precipitation, are related to a certain extent to the amounts of the various soil components, but these relations are far from clear and require further investigation.

Conclusion

It is concluded from the present study that phosphorus adsorption studied in soils before and after incubation did not exhibit a clear adsorption maxima if up to 50 ppm phosphorus solution is added. All the three soils before and after incubation do not follow Langmuir equation but Freundlich equation.

On ths basis of the present study, it is not worthwhile to have some recommendation, and further study is needed on phosphorus transformation taking into consideration all aspect of phosphorus cycles e.g respiration rate and enzymatic study alongwith field/pot experiment. Moreover, standardization of method, selection of the extractants, temperature, incubation period, all need a comparative and detailed study.

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GENE ACTION FOR SOME QUANTITATIVE CHARACTERS IN UPLAND COTTON (*GOSSYPIUM HIRSUTUM* L.)

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The study, includes a six-parent complete diallel cross of varieties/lines viz. CRIS-15, TH-3/83, TH-28/83, CRIS-9, NIAB-78 and Rehmani of *Gossypium hirsutum* L. It was initiated to determine the genetics of lint weight, seed index, staple length and seed cotton yield per plant. The WrVr graph showed that, lint weight and seed cotton yield were conditioned by over-dominance, while seed index and staple length by partial-dominance type of gene action. CRIS-9 and NIAB-78 x CRIS-9 appeared to be best general and specific combiners, respectively. Hence, they are recommended for future breeding programme.

Key words: Gene action, Genetics of cotton, Breeding potential.

Introduction

Cotton is one of the most important cash crop of Pakistan. It has played a pivotal role in the improvement of the economy of the country which cannot be ignored. Although, great advancements have been made in regard to improve the quality and yield of this crop, still there is a great potential in bringing the new varieties with better quality, high yield and better adaptability. Keeping these facts in mind, great responsibility falls upon plant breeders to work out plans for increasing the yield of cotton.

The success of plant breeding programme aiming at evolution of high yielding, better quality, responsive to high doses of fertilizer, disease and insect pest resistant varieties of cotton crop depends upon the selection of suitable parents to be utilized in any hybridization programme. Hence, the present study was under taken to determine the genetic system, affecting the economic traits of cotton plant, in order to facilitate to frame breeding strategy.

Materials and Methods

To assess the genetic architecture in upland cotton present studies were undertaken at the Agriculture Research Institute, Tando Jam during 1999-2000. Four cultures and two varieties of *Gossypium hirsutum* L., CRIS-15, TH-3/83, TH-28/83, CRIS-9, NIAB-78, and Rehmani were used.

Crosses were attempted in complete diallel fashion during 1998-99. The F_1 seeds along with their parents were sown in the field during kharif season of 1999-2000 in RCBD with 5 replications. Thirty and seventy-five centimeters distances were kept within and between the rows respectively. Recommended agronomic practices were performed at proper time. The data, regarding different characters were arranged and subjected to statistical analysis. Fisher's standard techniques of analysis of variance were applied to determine the differences among the treatments (Fisher 1958). Only the characters for which the genotypes showed diversity were assessed for genetics analysis. It includes diallel technique developed by Hayman (1954) and Jinks (1954 - 1956).

All the crosses were arranged into arrays in the form of diallel table and two statistics, the variance of arrays (Vr) and covariance of arrays and parental values (Wr) were calculated from the diallel table.

Information on gene interaction was obtained by plotting the covariance (Wr) of each array against its variance (Vr). The slope and position of regression line were fitted to array points within a limiting parabola.

Results and Discussion

The data presented in Table 1 revealed that the mean squares of all the traits, studied were highly significant, which indicated different heritage of genotypes.

Table 2 revealed that the CRIS-9 with the highest array mean (40.97 g) appeared to have better general combining ability for lint weight followed by CRIS-15 with array mean of (36.90 g)

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and within array, the cross NIAB-78 x CRIS-9 gave the highest value (51.2 g), indicating better specific combining ability.

Figure 1 shows that the regression line intersected the Wr-axis below the origin, showing over dominance type of gene action. From the position of the array points along the regression line revealed that NIAB-78 being nearest to the origin possessed maximum dominant genes for lint weight, while TH-28/83 had maximum recessive genes due to its farthest position. These results got support from the findings of White (1966), Verhalen *et al* (1971), Baker and Verhalen (1973). The results were in disagreement with those of Gururaja *et al* (1977), who reported that the character was controlled by partial dominance.

A perusal of Table 3 showed that the TH-28/83 with the highest array mean (8.26 g) appeared to have better general combining ability for seed index followed by CRIS-9 with array mean of (8.13 g) and within array, the cross Rehmani x TH-3/83 gave the highest value (138 g), indicating better specific combining ability.

Figure 2 indicates that the regression line intercepted the Wr-axis above the origin thereby, indicating partial type of gene action. The position of array points along the regression line, made it obvious that variety CRIS-9 had the maximum dominant genes due to its position closer to the origin, whereas,

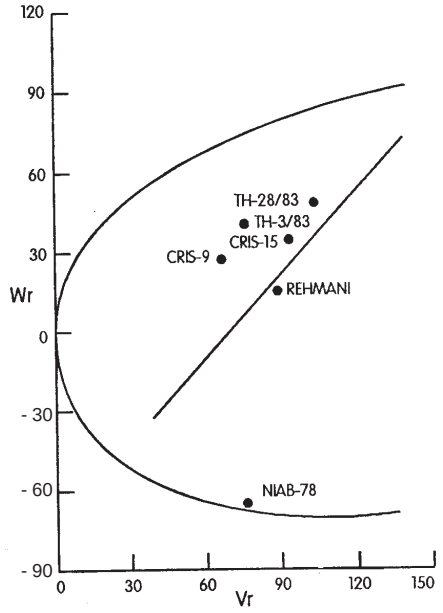

Fig 1. Wr/Vr graph for lint weight.

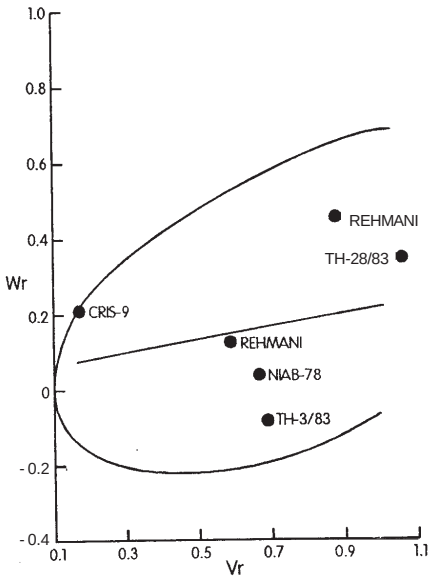

Fig 2. Wr/Vr graph for seed index.

Table 1

Analysis of variance (mean squares) for genotypes with respect to seed cotton yield and other traits in 6 x 6 diallel crosses of cotton

Source of variation	D.F	Lint weight	Seed index	Staple length	Seed cotton yield
Replication	4	509.53	1.44	0.655	3413.22
Treatments	35	404.58**	3.53**	3.54**	3030.89**
Error	140	192.7	0.93	1.46	1394.53

**Highly significant.

Table 2

6 x 6 Diallel. Average Lint weight (in grams) per plant variance (Vr) and covariance (Wr) for F_1 generation

Parents	CRIS-15	TH-3/83	TH-28/83	CRIS-9	NIAB-78	Rehmani	Wr	Vr
CRIS-15	42.22	33.32	35.44	50.00	32.86	44.54	35.71	84.23
TH-3/83	44.40	43.60	37.34	34.20	25.76	26.90	41.37	76.15
TH-28/83	26.50	25.10	17.58	31.30	49.80	26.30	49.81	114.48
CRIS-9	24.10	28.40	31.30	40.82	34.84	21.14	27.33	66.56
NIAB-78	47.60	42.20	49.96	51.20	32.20	40.70	- 64.61	76.00
Rehmani	36.60	46.10	28.52	38.28	43.80	38.44	15.14	88.96
Total	221.42	218.72	200.14	245.80	219.26	198.02	-	-
Array mean	36.90	36.45	33.36	40.97	36.54	33.00	-	-

TH-28/83 had the recessive genes due to its distant position. Gururaja *et al* (1977) and Sanyasi *et al* (1982) reported similar results. However, the study of Khan *et al* (1987) and Said Rehman and Khan (1988) was contradictory to these findings who reported complete or over dominance type of gene action for this character which might be due to differences in the genetic background of the parental varieties.

The highest array mean 28.55 mm was observed for Rehmani which proved better general combiner for staple length followed by CRIS-15, with array mean of 28.53 mm and within array, the cross CRIS-15 x Rehmani gave the highest value 29.6 mm, indicating better specific combining ability (Table 4).

The regression line intercepted the Wr-axis above the origin thereby, indicating partial type of gene action. The position of array points along the regression line made it obvious, that variety CRIS-9 had the maximum dominant genes due to its position closer to the origin whereas, NIAB-78 had the recessive genes due to its distant position. Findings of Verhalen and Murray (1969), are in accordance with these results. However, the studies of earliers (Verhalen and Murray 1967; Verhalen *et al* 1971; Chaudhry 1974; Gururaja *et al* 1977; Khan 1978; Khan *et al* 1980; Mirza and Khan 1984; Khan *et al* 1987; Khan *et al* 1990 and Shah 2000) are contradictory to these findings they reported complete or over dominance type of gene action for this character which might be due to differences in the genetic background of the parental varieties.

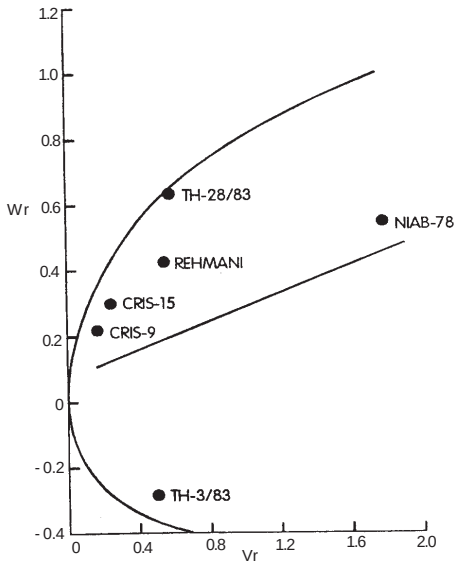

Fig 3. Wr/Vr graph for Staple length.

Table 3

6 x 6 Diallel. Average seed index (gm), variance (Vr) and covariance (Wr) for F_1 generation

Parents	CRIS-15	TH-3/83	TH-28/83	CRIS-9	NIAB-78	Rehmani	Wr	Vr
CRIS-15	8.02	8.56	9.56	7.46	7.80	7.92	0.4696	0.89
TH-3/83	8.16	7.74	8.80	8.00	8.06	8.72	-0.0897	0.70
TH-28/83	9.60	7.30	9.54	8.56	7.78	7.82	0.3559	1.68
CRIS-9	6.96	6.58	6.60	8.48	7.44	8.00	0.2172	0.18
NIAB-78	7.30	7.20	6.84	7.88	7.48	6.48	0.0487	0.67
Rehmani	8.58	8.74	8.20	8.38	9.64	8.40	0.1334	0.59
Total	48.62	46.12	49.54	48.76	48.20	47.34	-	-
Array mean	8.10	7.69	8.26	8.13	8.03	7.59	-	-

Table 4

6 x 6 Diallel. Average staple length (mm), variance (Vr) and covariance (Wr) for F_1 generation

Parents	CRIS-15	TH-3/83	TH-28/83	CRIS-9	NIAB-78	Rehmani	Wr	Vr
CRIS-15	28.80	28.10	28.60	27.90	28.50	29.60	0.30	0.25
TH-3/83	29.20	28.20	27.80	27.60	26.00	28.50	-0.79	0.52
TH-28/83	28.20	27.60	27.50	27.40	27.30	27.30	0.65	0.56
CRIS-9	28.00	27.70	27.50	27.80	26.60	28.40	0.22	0.17
NIAB-78	28.10	28.40	27.30	28.00	27.30	28.80	0.54	1.79
Rehmani	28.90	29.60	28.30	28.60	29.70	28.70	0.53	0.55
Total	171.20	169.60	166.00	167.30	165.40	171.30	-	-
Array Mean	28.53	28.27	27.67	27.88	27.57	28.55	-	-

Table 5

6 x 6 Diallel. Average yield of seed cotton per plant (in gm), variance (Vr) and covariance (Wr) for F_1 generation

Parents	CRIS-15	TH-3/83	TH-28/83	CRIS-9	NIAB-78	Rehmani	Wr	Vr
CRIS-15	115.08	94.00	90.44	136.70	88.30	112.56	405.56	560.86
TH-3/83	126.50	126.50	100.30	88.00	72.50	72.50	454.31	893.39
TH-28/83	74.20	61.40	48.70	82.30	125.20	69.70	415.30	695.87
CRIS-9	65.00	77.20	97.20	104.20	88.80	56.70	168.31	795.88
NIAB-78	102.92	116.40	128.70	148.80	89.80	11.00	-448.37	388.65
Rehmani	102.60	138.50	79.00	87.40	115.80	97.40	96.61	552.87
Total	586.30	614.00	544.34	647.30	580.40	519.86	-	-
Array mean	97.72	102.33	90.72	107.88	96.73	86.64	-	-

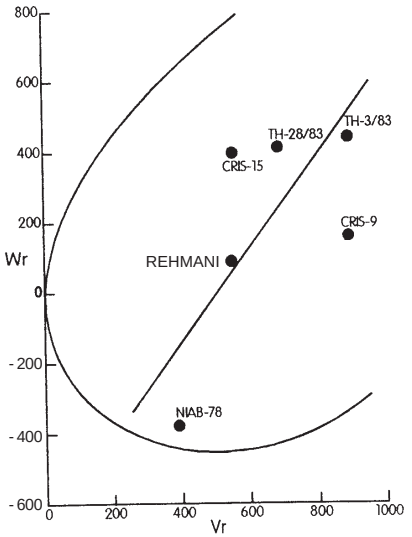

Fig 4. Wr/Vr graph for seed cotton yield.

A perusal of Table 5 showed that the CRIS-9 with the highest array mean (107.88) had better general combining ability for yield of seed cotton followed by TH-3/83, with array mean of (102.33 g) and within array, the cross NIAB-78 x CRIS-9 gave the highest value (148.8 g), indicating better specific combining ability. This showed that among the entire cross combinations studied these parents NIAB-78 x CRIS-9 had best specific combining ability. Hence, this combination might exhibit superior hybrids in later segregating generations for this character.

It is observed from the Fig. 4 that the regression line intercepted the Wr-axis below the origin, signifying the over dominance type of gene action. The position of array points along the regression line revealed that NIAB-78 had the maximum dominant genes being nearest to the origin, while TH-3/83 possessed the maximum recessive genes due to its farthest position. Similar results have been reported by the following (Marani 1961; Verhalen *et al* 1971; Mirza and Khan 1974; Patil 1977; Salem *et al* 1984; Sadykhova 1986; Jagtab and Kohle 1987; Said Rehman and Khan 1988; Khan *et al* 1990). However, White and Kohel (1964) and Ramey and Miller (1966) have reported partial dominance. The difference might be attributed to different genetic stock and ecology.

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Studies on Detoxification of Feeds and Feed Ingredients

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Different feed ingredients i.e. wheat, sorghum, maize, corn gluten meal, rice bran, cotton seed meal, mustard seed meal, sunflower meal, soy bean meal, sesame meal, peanut meal and broiler feeds were procured from local market and analyzed for chemical composition and toxic factors. The oilseed cakes and rice bran meal were treated to eliminate the toxic factors. Detoxification of feed ingredients resulted in decrease in gossypol from 0.31 to 0.05% in cotton seed meal, allylisothiocyanate from 1.55 to traces in mustard seed meal, chlorogenic acid from 3.20 to 0.95% in sunflower meal and trypsin inhibitor from 72.10 to 17.20 mg/g in soy bean meal. Phytic acid contents in oilseed cakes i.e. cotton seed, mustard seed, sunflower, soybean, and rice bran meals before and after treatments were reduced from 2.91 to 0.43, 3.54 to 0.40, 2.84 to 0.52, 3.22 to 0.64 and 2.60 to 0.42%, respectively.

Key words: Feed ingredients. Toxic factors, Cereals, Phytic acid.

Introduction

Pakistan produce around 20844 thousands metric tonnes of cereals which are used as food and feed products (Economic Survey 1999-2000). Only a small fraction of low quality broken cereals or their useless byproducts i.e. wheat bran, rice bran, rice polishing etc. are used as poultry or animal feed ingredients. Similarly 2016 thousands tonnes oilseeds are produced annually (Economic Survey 1999-2000). The cake left after extraction of edible oil, is a potential source of protein, carbohydrates, minerals, etc (Aherne and Kennelly 1983) but has limited use in poultry feed (Christian 1958; Bernard and Goloblatt 1980; Aherne and Kennelly 1983; Shah and Mahmood 1986) due to the presence of toxic and antinutritive factors such as gossypol (cotton seed) glucosinolate (mustard rapeseed), trypsin inhibitor (soybean) and chlorogenic acid (sunflower seed). The left over cakes of these oil seeds and cereals also contain antinutritive factors like phytic acid. Phytic acid is myo-insoitol - 1,2,3,4,5,6 hexanis-dihydrogen phosphate. In most seeds it serves as primary phosphorus and myo-inositol reserve. Phytic acid forms chelating compounds with proteins and minerals and decrease their solubility, functionality, digestibility and physiological availability, while appropriate treatments have been suggested to reduce or eliminate toxic and antinutritional compounds of various feed ingredients (Maga 1982; Cosgrove 1980; Rodriguez *et al* 1985).

Present investigations were carried out to determine toxic and antinutritive factors in oil seed meals and efforts were made to reduce or eliminate these factors from the meals to be used in poultry feed and to produce a toxin-free poultry feed.

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Materials and Methods

Four broilers feed samples and different feed ingredients i.e. oilseed cakes, cereals, rice bran, etc were produced from local market.

Processing. Cotton, mustard, sunflower, soybean, sesame, peanut seed cakes and rice bran were ground to fine powder (60 mesh) and placed in soxhlet extractor (Quickfit England) for 20 hr with *n*-hexane for reducing oil contents to the minimum (1 ± 0.5%). The meals so obtained were dried, by placing them in open air and finally in an oven at 60 ± 2˚C followed by grinding to fine powder.

Detoxification procedure. 1. *Cotton seed meal*: Five hundred grams cotton seed meal were mixed with a solution containing 1.0% $Ca(OH)_2$ + 0.15% $FeSO_4$ to eliminate free gossypol (Saqib *et al* 1997) and 3.0% NaCl (w/w of meal) was also added later on to help in leaching out phytic acid (Niazi *et al* 1988). The cotton seed meal mix was boiled for 30 min and filtered. The residue was dried in an oven at 80 ± 2˚C and ground to 80 mesh size.

2. *Mustard seed meal*: Five hundred grams mustard seed meal were dipped in water (1:5 w/v) at 55˚C for 45 min followed by steam stripping to remove toxic factor allylisothiocyanate (Mustakas *et al* 1965). Then 3.0% NaCl (w/w of meal) was added to the mixture and its pH was adjusted to 5. The mixture was kept at the same temperature for one hour, followed by filtration (Niazi *et al* 1988). The residue was dried in an oven at 80 ± 2˚C and grounded to 80 mesh size.

3. *Sunflower meal*. Five hundred grams sunflower meal seed were soaked in 0.2% sodium bisulphate for 20 min and kept for

one hour at ambient temperature (Bau *et al* 1983) followed by leaching with 3.0% NaCl solution (1500 ml) at 55°C. The pH was adjusted to 5 and kept for one hour. The slurry was filtered and the residue was dried and powdered as mentioned above.

4. *Soybean meal*. Soybean meal was treated for reduction of trypsin inhibitor and phytic acid contents (Sathe and Salunkhe 1981). It was processed as described for sunflower meal except that 0.25% $NaHCO_3$ was used instead of 0.20% $NaHSO_3$.

5. *Rice bran meal*. The rice bran meal was wetted with deionised water (1:2 w/w basis). Its pH was adjusted to 5 with 1N HCl and autoclaved at 1 kg/cm (120°C) for 30 min to inactivate the enzyme lipase and also dephosphorylase present in it, followed by drying at 80°C and grinding to 80 mesh size (Niazi *et al* 1997).

6. *Analytical methods*. Standard procedures previously described were used to determine concentration of gossypol (AOAC 1990), allyisothiocyanate (Wetter 1955), chlorogeneric acid (Bau *et al* 1983), trypsin inhibitor (Eskin *et al* 1978) and phytic acid (Wheeler and Ferrel 1971). Proximate composition (moisture, fat, crude protein, crude fibre, ash and nitrogen free extract) were determined according to standard procedures of AOAC (1990).

Results and Discussion

Proximate composition of oilseed meals and rice bran meal (Table 1) indicated that high protein (39.21 to 53.22%), nitrogen free extract-NFE (31.22% to 42.77%) and ash (6.40% to 8.12%) contents are essential ingredients to be added into feeds. As these ingredients are potential source of energy and minerals, required for animal and poultry feed preparation. Furthermore, elimination or reduction of toxic and antinutritive factors of these would also improve the nutritive value of feeds.

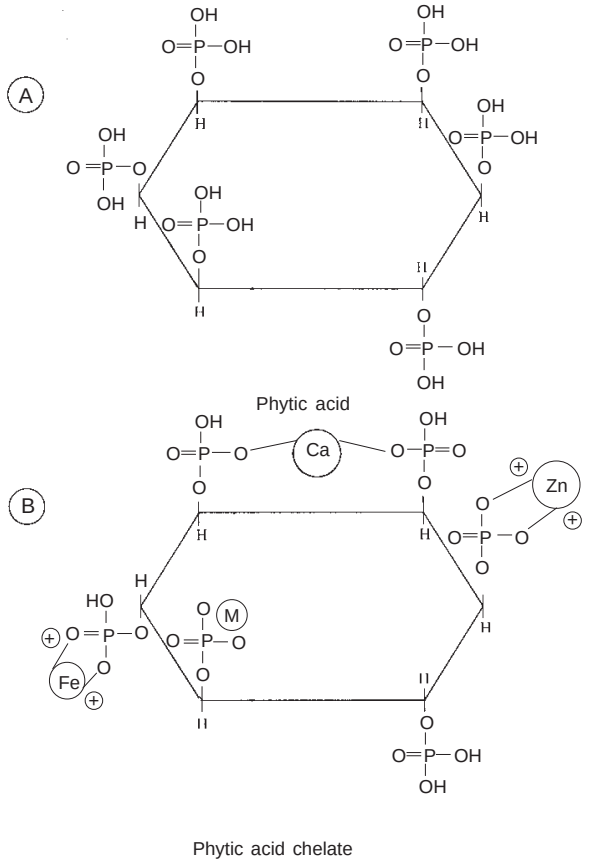

Fig 1. Structures of phytic acid (A) and phytic acid chelate (B) at neutral pH

Table 1
Proximate composition* of oil seed meals and cereals

Oilseed Meals	Moisture (%)	Crude protein (%)	Fat (%)	Crude Fibre (%)	Ash (%)	NFE (%)
Cotton seed meal**	7.75	39.21	1.91	7.11	6.40	35.37
Mustard seed meal	5.80	41.11	1.50	10.90	6.81	39.68
Sunflower seed meal**	6.23	51.54	2.12	6.92	8.11	31.22
Soybean seed meal	8.84	40.50	1.90	6.71	8.12	42.77
Sesame seed meal	9.15	53.22	1.41	10.83	5.80	38.74
Wheat	8.50	12.50	2.58	2.70	1.90	71.52
Sorgham	9.10	11.75	2.05	2.50	2.10	72.50
Maize	9.00	8.80	3.00	2.40	2.15	74.65
Corn gluten meal 60%	8.15	59.50	3.50	1.00	1.50	26.35
Rice bran	7.00	15.50	3.00	14.90	13.50	46.10
Broiler feed	10.50	20.70	3.00	2.80	8.10	54.90

* On dry matter basis; ** Decorticated/dehulled; NFE: Nitrogen free extract.

Amount of different toxic material such as gossypol, allylisothiocyanate, chlorogenic acid, trypsin inhibitor and antinutritive factors i.e. phytic acid in oil seed meal and creals are shown in Table 2. Toxic and antinutritive factors determined in different feed ingredients showed that cotton, mustard, sunflower, soybean meals contained 0.31% gossypol, 1.55% allylisothiocyanate, 3.20% chlorogenic acid, 72.10 mg/g trypsin inhibitor, respectively. Incorporation of these ingredients in poultry feed at higher level would certainly exert adverse effect on the nutritional value of feeds. Thus, it is essential to detoxify these feed ingredients.

Detoxification of oil seed meals and rice bran meals did show losses in proteins but overall recovery of detoxified oil seed meals (81.83 to 85.70%) and rice bran meal seemed economically, acceptable (Table 2). Detoxification effectively reduced gossypol (0.05%). allylisothiocyanate (traces), chlorogenic acid (0.95%). trypsin inhibitor (17.20 mg/g), and phytic acid contents (0.40% to 0.64%) to safer limits. Losses in weight of protein which occurred during different treatments appeared to be much less as compared to the nutritional improvement in oilseed meals. But the other feed ingredients when supplemented in poultry feed, increased over-all performance.

All the oil seed meals and rice bran meal contained 2.60 to 3.54% phytic acid, maximum being in mustard and minimum in rice bran (Table 2). Cereals i.e. wheat, sorghum and maize grains contained 1.08 to 1.24% phytic acid, whereas cereal by products i.e. rice bran meal and corn gluten meal which are used as poultry feed ingredients contained 1.52% to 1.94% phytic acid, respectively. The results are in agreement with the findings of Eardman (1979) and Niazi *et al* (1997). Broiler feeds available in local market showed on an average 1.81% phytic acid (Table 2).

The structure of phytic acid and phytic acid chelate at neutral pH is shown in Fig 1. It is apparent that various cations could strongly chelate between two phosphate groups depending upon the strength of various metal ions. Similarly, proteins form loose complexes with phytic acid which affect their bioavailability as indicated by Eardman (1979).

Table 2
Antinutritive factors in oil seeds and cereals and detoxified oil seed meals

Sr. No.	Oil seed meals untreated/treated	Crude Protein (%)	Gossypol (%)	Allyliso-thiocyanate (%)	Chlorogenic acid (%)	Trypsin inhibitor (gm/g)	Phytic acid (%)	Recovery (%)
1.	Cotton seed meal	39.12	0.31	-	-	-	2.91	-
2.	(i) 1% $Ca(OH_2)$ + 0.15% $FeSO_4$ (ii) 3% NaCl	36.40	0.05	-	-	-	0.43	85.70
3.	Mustard seed meal	41.12	-	1.55	-	-	3.54	-
4.	(i) Enzymic.Detoxified (ii) 3% NaCl	38.52	-	Traces	-	-	0.40	81.83
5.	Sunflower seed meal	51.44	-	-	3.20	-	2.84	-
6.	(i) 0.2% $NaHSO_3$ (ii) 3% NaCl	45.81	-	-	0.95	-	0.52	82.64
7.	Soybean seed meal	40.52	-	-	-	72.20	3.22	-
8.	(i) 0.25% $NaHCO_3$ (ii) 3% NaCl	38.24	-	-	-	17.20	0.64	81.85
9.	Rice bran meal	12.20	-	-	-	-	2.60	-
10.	(i) Meal water (1:2), pH:5 (ii) Autoclaving 38 min. 1 kg/cm_2 (iii) 3% NaCl	12.05	-	-	-	-	0.42	-
11.	Wheat	12.50	-	-	-	-	1.24	-
12.	Sorghum	11.75	-	-	-	-	1.08	-
13.	Maize	8.80	-	-	-	-	1.15	-
14.	Corn gluten meal	59.50	-	-	-	-	1.94	-
15.	Rice bran	15.50	-	-	-	-	1.52	-
16.	Broiler feed	20.70	0.009	0.075	-	1.70	1.81	-

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Short Communication

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EFFECT OF *RAPHANUS SATIVUS* LINN OIL ON RABBIT SKIN

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Raphanus sativus Linn is commonly known as radish, (Muli safed) belongs to family Cruciferae and cultivated through out subcontinent Indo Pak (Nadkarni 1954). The plant is an excellent source of vitamins B and C. This plant is used as purgative, stimulant, antiscorbutic, diuretic and lithotryptic. The seeds has been used as emmenagogue and in treatment of gonorrhea and cancer (Watt *et al* 1962).

Seeds of radish are expectorant, diuretic, laxative, carminative (Kirtikar and Basu 1933; Chopra *et al* 1958). Seeds and root contains a fixed oil, essential oil, a sulphurated volatile oil which resembles mustard seed oil. This oil contains sulphur and phosphoric acids (Nadkarni 1954; Watt *et al* 1962).

The crude enzyme chitanase purified from radish seed found to posses relatively, high chitin hydrolysing activity. (Kondo *et al* 1997) seed contains β sitosterol (Wang *et al* 1997). Due to above mentioned importance, the radish seeds were chosen for further pharmacological studies.

The *Raphanus sativus* seeds of the best quality (one Kg) were purchased from local market and identified by the Pharmacognosy section of these laboratories. Seeds were washed thoroughly with distill water and dried in open air at room temperature at 37°C for 24 hrs.

Extraction of oil. The wash and dried seeds (50 g) were subjected to a kitchen chopper (10,000 rpm). Oil from the seeds powder extracted in a soxhlet extractor with pet- ether (B.P 40 - 60°C) 200 ml. The solvent was removed under reduced pressure to furnish oil which was of golden yellow color (14.87g, 29.74%) (Zahra *et al* 1999).

Acute toxicity test on Albino mice. The acute test (72 hours) of *Raphanus* oil was determined by the administration of oil via oral route in albino mice (25 - 30g). *Raphanus* oil test material was administered in doses of 1600, 1800 and 2100 mg/kg body wt to mice of groups 1, 11 and 111; while group IV was maintained as standard and received the olive oil. Group V kept as control and received the normal saline. Each group comprised of six animals of either sex, which were observed for one month, there was no extra ordinary change in the physical condition and then autopsied to see any gross changes in various organs i.e. heart, lung, liver, stomach, spleen, G.I.T., kidneys, ovaries and testes. *Raphanus sativus* Linn given by oral route was found to be nontoxic (Loomis 1978).

Effect of Raphanus oil on rabbit skin. Rabbits having an average weight of 2 kg – 2.5 kg were used. The skin was shaved off. The diameter of shaved portion was 2.5 ± 0.2 inches. The shaved portions were washed with lukerious water to remove dust and other adhering particles. Skin tests were carried out on either side (Loomis 1978). The animals were photographed before and after completion of observation period (Table 1).

The animals were kept in separate cages for local application for 30 days and considered as test group. The test oil 0.1 ml was taken from the measuring dropper and rubbed with spatula on either side of the rabbit skin. *Raphanus* oil was absorbed from the skin, there was no protection over the shaved area. No precautions were taken to prevent the animals from ingesting the oil (Fig 1). Olive oil was used as standard on another group of animals taken as standard group because it is a well known emollient having no toxic effect, (Fig 2) (Gernnaro 1985; Maryam *et al* 1993).

Some naturally wounded rabbits were found and their wound treated with *Raphanus sativus* Linn oil in same

Table 1
Effect of *Raphanus sativus* oil on rabbit skin

Group	No. of rabbit	Age (Month) per day	Dosage (In ml) Oil (days)	No. of Application per day	Periods experiment (days)	Amount of drug applied in 30 days (ml)	Toxic effects	Resuts
Test	6	3	0.1 (*Raphanus* oil)	2	30	12.0	Nil	+
Standard	6	3	0.1 (olive)	2	30	12.0	Nil	+
Control	6	3	Nil	2	30	-	Nil	+

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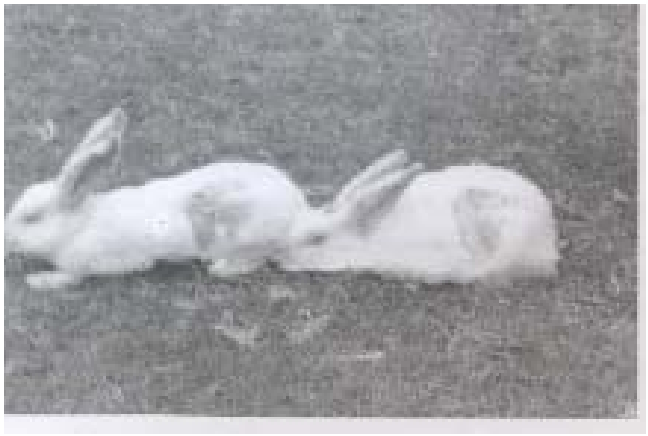

Fig 1. Test group after 30 days treatment with *Raphanus* oil

Fig 2. Standard group after 30 days treatment with olive oil.

Fig 3. Naturally wounded rabbit.

protocol (Fig 3). Recovery took place after 15 and 30 days (Fig 4 and Fig 5).

The results of the effect of *Raphanus* oil on rabbit skin has been compared with olive oil and control in Table 1. During 30 days of treatment no animal showed any injurious effect i.e. redness, swelling, cracking or itching on skin during the

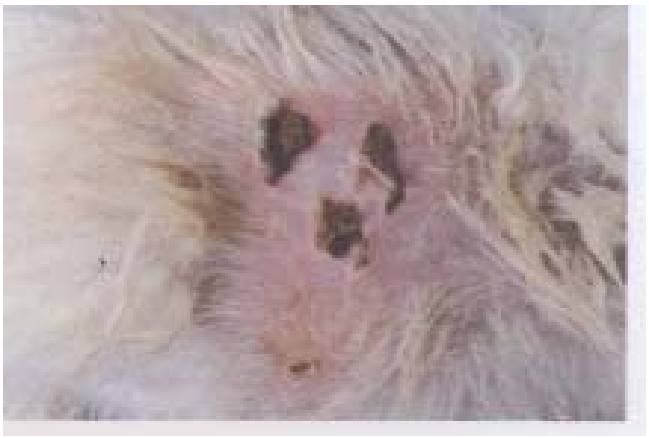

Fig 4. Wounded treated with *Raphanus* oil after 15 days.

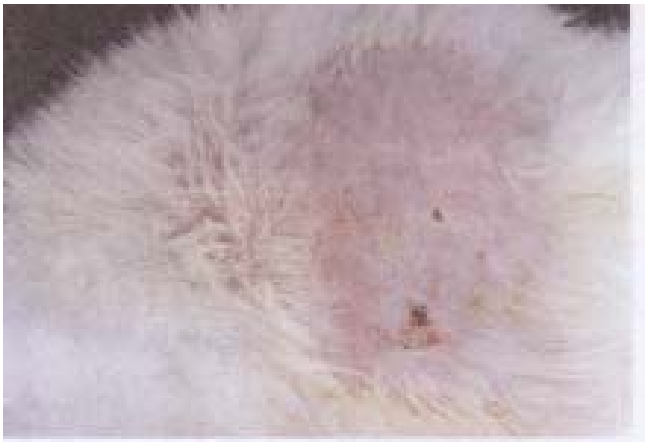

Fig 5. Wound after treatment of 30 days with *Raphanus* oil.

observation period. The results indicate, that performance of both the oils is almost identical. These tests asses the dermal toxicity on animal skin.

Key words**:** *Raphanus sativus* Linn seed oil, Toxicity, Wound, Healing.

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Commercial Extraction and Resolution of Silymarin Isomers

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A simple and economical procedure for the large-scale extraction of silymarin from the dried seeds of *Silybum marianum* has been developed. After harvest the cellular and acellular constituents of the seeds are biodegradable. Using the fresh seeds, a viable procedure has been perfected for the extraction of silymarin (yield 1.47 %). The product appeared as a mixture and was resolved into the isomers by centrifugal chromatography. The identity of the product and its isomers were confirmed through comparison with standards using mp, UV, IR, ^{1}H NMR and Mass spectrometric data and 2,4, dinitrophenylhydrazone assay.

Key words**:** *Silybum marianum* seeds, Silymarin, Silybin, Silydianin, Silychristin.

Introduction

Silybum marianum a medicinal herb has been widely used in the European traditional medicine (Eichler and Hahn 1949). Extracts prepared from the roots and seeds have been used in the treatment of liver diseases, disorders of bile duct and spleen. (Sonnenbichler *et al* 1998) Nowadays, silymarin, the purified compound of the seeds and its major isomer silybin are used in the manufacture of therapeutic products administered against liver diseases, jaundice, and gallstones (Wagner *et al* 1968; Wagner 1973). Despite the abundance of the indigenous species, the seeds have not been studied as a source for silymarin or its isomers silybin, silydianin and silychristin. The present studies are undertaken for the development of a commercially viable process for the extraction of silymarin from the seed powder and preparation of the isomers.

Materials and Methods

Materials. Ripe fruits of *Silybum marianum* L. were collected from the experimental garden of PCSIR Laboratories Peshawar. Silica gel 60 PF 254+366 was used for resolution of the isomers. Solvents and chemicals were BDH Laboratory analytical grade reagents. Melting point was determined using Griffin MFB S90 010T Electrothermal apparatus. Resolution of silymarin isomers was attempted using Harrison Research Model Chromatotron.

Extraction of silymarin. Dried capitulums of *Silybummarianum* were thrashed, seeds were separated and powdered material (10 kg) was extracted three times with hexane (25 l) through solvent recycling at room temperature for 10 h. Concentration and removal of hexane under reduced pressure yielded 2.2 kg oil.

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The defatted material (7.7 kg) was then extracted four times with ethanol (20 l) by percolation and recycling at room temperature for 12 h. The combined alcoholic extract (15 l) was filtered, concentrated to one litre under reduced pressure and gradually added to water (5 l) under intensive stirring. The product salted out from the aqueous extract with sodium chloride (400 ml, 25%). The precipitated material was filtered, washed with water, dried in an electric vacuum oven and powdered (Fig 1). The yield of the crude material was 204g.

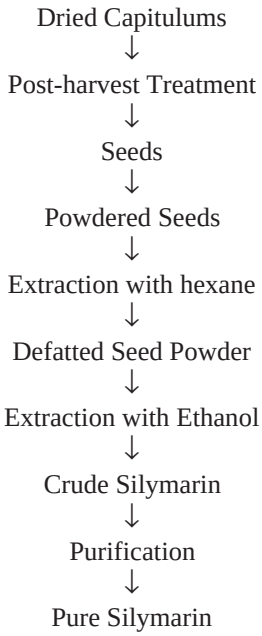


Fig 1. Flow Chart for the Extraction and Purification of Silymarin

The crude product was dissolved in absolute ethanol (1 l) with stirring and shaking. The solution was filtered and then cooled

to 10°C. The resultant turbid solution was filtered and the filtrate dried under reduced pressure, residue obtained was dissolved in acetone (400 ml), the final yield of silymarin was 1.47%.

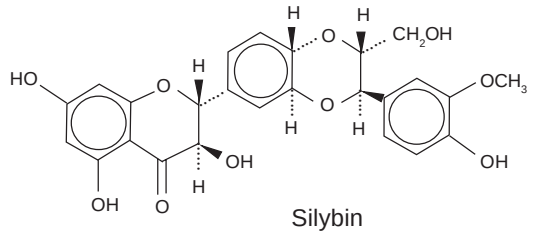
Analysis Techniques. The UV spectra in methanol were recorded on a Hitachi Model U-2000 Instruments. Infrared spectra were recorded using a Pye-Unicam SP 3-100 model. ^{1}H,^{13}C-NMR spectra were recorded with the help of Bruker 400 MHz spectrophotometer using tetramethylsilane (TMS) as internal standard. Deuterated methanol and acetone were used as solvent. Mass spectra was obtained at the HEJ Research Institute of Chemistry, University of Karachi, Pakistan. Melting point of the product was 170-175°C, UV: 288, 326 nm, IR (KBr disc): 3380, 3450, 1752, 1615, 1507, 1262, 1137 cm^{-1}. Assay of the product was performed according to Wagner *et al* (1968, 1974). TLC examination of the product against reference silymarin, carried out in a solvent system hexane, chloroform, methanol (4: 3: 1.8) showed the presence of three components.

Resolution of silymarin. Fractionation of silymarin components was carried out using Harrison Research Mode 7924T Chromatotron. Sorbent layer of 1 mm thickness was prepared from silica gel 60 PF 254+366. The layer was equilibrated with the elution system comprising of hexane, chloroform and methanol (3: 2.5: 0.75) Purified silymarin (100 mg) was dissolved in methanol (0.5 ml) and the solution applied without loss to sorbent layer as a compact ring. Washing of the sample was performed at a flow rate 2 - 4 ml/min, whilst revolution/min was maintained at 750. Separation of the components was monitored under UV light. Ten fractions, 10 ml each, were collected and compared by TLC against standard silybin, silydianin and silychristin. The yield of each compound recovered was 55%, 34% and 11 % respectively.

Spectroscopy. *Compound:* 1 (yield 40 mg, m.p. 180 °C dec) UV (Methanol) λ max. 288 nm ^{13}C-NMR (CD_3OD, DMSO-d_6) δ196.6, 165.9, 162.5, 161.6, 146.8, 146.2, 142.9, 142.5, 129.3, 126.7, 120.9, 120.1, 115.8, 114.7, 111.0, 99.8, 95.5, 94.5, 82.0, 77.6, 75.3, 71.0, 59.8, 55.2. MS m/z: 482 M^+, (Calcd for $C_{25}H_{22}O_{10}$), and other major fragments at 180, 162, 138 and 124.

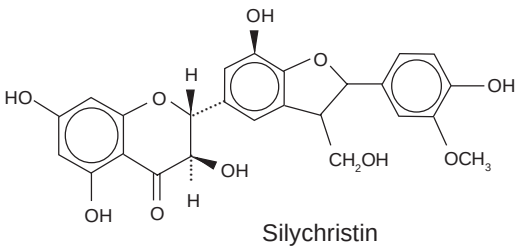
Compound. 2 (yield 25 mg, m.p.-191°C) ^{1}H-NMR (acetone-d_6, TMS, 100MHz) δ2.97 (1H, m, H_γ), 3.27 (1H, d, H_α), 3.40 (1H, m, H_β), 3.70 (1H, q', $H_{3'}$), 3.87 (3H, s, OCH_3), 3.88 (1H, d, H_6), 4.32 (1H, q, H_D), 4.63 (1H, d, H_3), 4.98 (1H, q, H_2), 6.06 (2H, s, H_6, H_8), 6.27 (1H, d, $H_{2'}$), 6.73-7.00 (3H, m, $H_{2''}$, $H_{5''}$, $H_{6''}$), 11.70 (1H, s, H_5-OH). ^{13}C-NMR (CD_3OD, DMSO-d_6) δ201.80, 196.40, 166.80, 163.30, 161.90, 147.00, 144.90, 139.40, 132.90, 124.00, 120.20, 114.80, 112.40, 100.20, 96.60, 96.10, 95.00, 81.60, 72.70, 70.80, 55.40, 53.30, 46.60, 46.00, 44.00. MS m/z: 482 M^+, (Calcd for $C_{25}H_{22}O_{10}$), and other major fragments at 302,180.

Compound. 3 (yield 8 mg, m.p. 174-176°C) UV (Methanol) λ max. 288nm. ^{1}H-NMR (acetone-d_6, TMS, 100MHz) δ3.61 (1H, m, H_β), 3.84 (H, S, OCH_3), 3.86 (1H, m, H_7), 3.92 (1H,



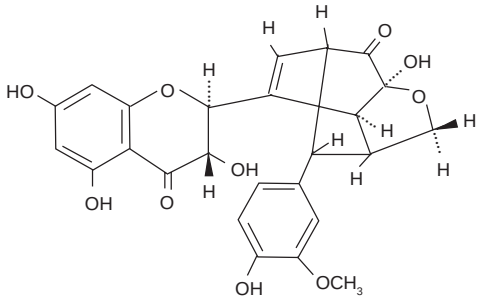
Silybin

Structural formula of silybin



Silychristin

Structural formula of silychristin



Silydianin

Structural formula of silydianin

Scheme 1. Structure of the active constituents of Silymarin.

dd, H_7), 4.64 (1H, d, H_2), 5.05 (1H, d, H_3), 5.59 (1H, d, H_α), 5.96 (1H, d, H_6), 6.00 (1H, d, H_8), 6.83 (1H, d, $H_{5''}$), 6.93 (1H, dd, $H_{6''}$), 7.00 (1H, d, $H_{2'}$ or $H_{6'}$), 7.03 (1H, d, $H_{2'}$ or $H_{6'}$), 7.10 (1H, d, $H_{2''}$). ^{13}C-NMR (CD_3OD, DMSO-d_6) δ198.20, 168.70, 165.20, 164.40, 149.10, 147.50, 142.10, 134.70, 131.50, 130.10, 119.80, 117.00, 116.60, 116.20, 110.70, 101.80, 97.40, 96.30, 89.10, 85.20, 73.70, 64.80, 56.40, 55.40. MS m/z: 482 M^+, (Calcd for $C_{25}H_{22}O_{10}$), and other major fragments at 302, 180, 162, 137.

Results and Discussion

The isolation of phytopharmaceuticals in pure form from plants is often carried out with multistage extraction and purification procedures (Meilroy 1951; Trease and Evans 1983). Normally neutral solvents are used for separation because they tend to hydrolyze. After extraction from plants, resolution of the complex mixture required the application of a number of other techniques due to the co-extracted proteins, fats, oils, carbohydrates, pigments and other constituents. The undesired components are usually precipitated from the extract by the addition of specific reagents.

On account of the therapeutic and economic importance of silymarin, specific procedures have been reported on the industrial extraction of the plant but the information is mainly confined to patent literature. In the present work for the selective extraction of the pigments, fats, oil and resinous substances from the seeds powder, hexane was used. During extraction heating of the seed powder was avoided on account of decomposition of the labile compounds. In addition, concentration and purification of the hexane extract performed under reduced pressure at 30°C, yielded 22% oil. Evaluation of which on TLC showed presence of oleic acid, sitosterol, stigmasterol and cholesterol. The results were consistent with earlier findings of Hammouda *et al* (1994).

Commercial scale purification of phytochemical often presents several problems. When it appears that a plant derived product may contain several adhering constituents, the choice of obtaining the desired constituent becomes very significant. For selective extraction of silymarin from the seed powder extraction at room temperature (25°C) and concentration, purification of the extracted constituents under reduced pressure at 35°C, prevented the decomposition of the coextracted components and adhering impurities. Initial separation of macro impurities was achieved at 15°C by solubilisation and sedimentation. It was thought that if particles of varying size were allowed to suspend in alcohol, then the force of gravity would appear to be the decisive factor for their sedimentation. As a result the large and denser molecules quickly settled on the bottom and were removed by filtration. Microfine particles appeared completely dispersed in the solvent phase. Lowering the temperature to 10°C coagulated and facilitated their removal from the solvent phase. With the removal of adhering impurities purification of silymarin appeared very simple. The yield of pure silymarin was 1.47% and was close to the reported values. (Kurkin *et al* 1996). Assaying the material as 2,4-dinitrophenylhydrazone of silybin confirmed the presence of 98 percent flavonoids. As compared to patent procedures for extraction of silymarin (Wagner *et al* 1968; Wagner 1973; Wagner *et al* 1974; Kurkin *et al* 1996) the method described was simple efficient and feasible.

During centrifugal separation of the isomers, the selection of eluent was made on the basis of Rf values, which were kept below 0.5. Under the chosen experimental conditions (Rf, flow rate, r.p.m and temp.) the components were easily separated. Physicochemical characteristics, m.p, TLC, UV, IR, ^{1}H-NMR, ^{13}C-NMR, and Mass spectrometric data of component 1,2 and 3 were identical with those reported in literature for *silybin, silydianin* and *silychristin* (Pelter and Hansel *et al* 1968; Abraham *et al* 1970; Wagner *et al* 1971; Szilagi *et al* 1981). The yields of the isomers were consistent with the reported values (Kurkin *et al* 1996). Compared to the reported resolution procedures, (Hostettmann *et al* 1986) the technique applied for separation of the silymarin isomers was convenient.

The methodology applied for the commercial extraction of silymarin and its isomers is simple, efficient and designed for minimum equipment. The products are of therapeutic and commercial significance and have been used in the preparation of hepatoprotective drugs marketed under various trade names.

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EXAMPLES

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- Nitrogen over-use, under-use and efficiency

Symposia:
- Farming systems, cropping sequences, and monocultures
- Conservation and reduced tillage systems
- Turfgrass: the most visible crop
- Future of Asian cropping systems
- Biocide dependence and resistance
- Nutrient recycling and balance in cropping systems
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