ISSN 0973 – 9874 J.Pharm.Chem CODEN: JPCOCM

Journal of Pharmacy and Chemistry

(An International Research Journal of Pharmaceutical and Chemical Sciences)

ISSN 0973 – 9874 J.Pharm.Chem CODEN: JPCOCM

Journal of Pharmacy and Chemistry

(An International Research Journal of Pharmaceutical and Chemical Sciences)

Volume 3 • Issue 4 • October – December 2009

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VIEWS

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Synthesis of some new (1, 3, 4) oxadiazino-[5, 6-b] indole derivatives and their biological activity

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ABSTRACT

Twenty one New 2-[(benzalamino-4-hydroxybenzyl) (1,3,4)-oxadiazino[6,5-b]] Indole derivatives (**V**) have been synthesized by condensing 2-Amino-4-[(1,3,4)oxadiazino[6,5-b]indole-3-yl]-phenol (**IV**) with various aromatic aldehydes. The intermediates, on the other hand, have been synthesized by the cyclization of 3-Amino-4-hydroxy-benzoic acid (2-oxo-1,2-dihydro-indol-3-ylidene)-hydrazide (**III**) in presence of Concentrated H_2SO_4. The title compounds have been purified and characterized by their analytical and spectral data. They have screened for their antimicrobial activity and the results are presented.

Key words: (1,3,4)oxadiazino-[5,6-b] Indole, Isatin derivatives, Antimicrobial activity.

Introduction

It is known from the literature that Indole derivatives exhibit varied biological and pharmacological properties [1-7] viz. antimicrobial, antiviral, anti neoplastic, analgesic, CNS activities. In view of these observations the synthesis of new (1,3,4)oxadiazino-[5,6-b]- indole derivatives(V) has been carried out (Scheme-1)

For this purpose the required indole-2,3-diones (I) were prepared and condensed with 3-amino-4-hydroxybenzoicacidhydrazide(II) in ethanol to get the respective 3-Amino-4-hydroxy-benzoic acid (2-oxo-1,2-dihydro-indol-3-ylidene)-hydrazide (III). These compounds were cyclized using concentrated sulfuric acid to get respective 2-Amino-4-[(1,3,4)oxadiazino[6,5-b]indole-3-yl]-phenol (**IV**)These compounds were refluxed with aromatic aldehyde, ethanol and few drops of acetic acid to get the title compounds as shown in Scheme – 1. The compounds were characterized by their physical, analytical and spectral data (IR and NMR, MASS). The physical and analytical data is presented in Table 1 and II and data on antimicrobial activities is presented in Table III.

Methods

Experimental:

The melting points (in^{0}C) were recorded in open capillaries using Toshniwal melting point apparatus and are uncorrected. The purity of the compounds was checked by TLC using ethyl acetate and Chloroform (0.5ml: 1.5ml) as solvent system and Iodine vapours as visualizing agents. The IR spectra were recorded on Perkin-Elmer Infracord-283 spectrophotometer. PMR spectra were recorded on OMEGA-500 mHz spectrophotometer using TMS as an internal standard. Mass spectra wre recorded by the direct inlet method on FINNINIGAN MAT -90 in the EI mode.

General Procedure:

Isatins (I), 3-amino-4-hydroxybenzoic acid hydrazide (II) were synthesized by the methods available in literature.

3-Amino-4-hydroxy-benzoic acid (2-oxo-1,2-dihydro-indol-3-ylidene)-hydrazide (III):

An appropriate Isatin (I, 0.01 mol) was heated under reflux, in ethanol (50 ml) with 3-amino-4-hydroxybenzoic acid hydrazide (II, 0.01 mol) for 1.5 hrs; the product thus separated was filtered and purified by recrystallization from suitable solvents. The physical constants were compared with the literature values.

2-Amino-4-[(1,3,4)oxadiazino[6,5-b]indole-3-yl]-phenol (IV):

An appropriate 3-Amino-4-hydroxy-benzoic acid (2-oxo-1,2-dihydro-indol-3-ylidene)-hydrazide (III, 0.0.1 mol) was dissolved in 10 ml of concentrated Sulphuric acid. The reaction mixture was kept aside for 2 hours, poured on to crushed ice and neutralized with sodium bicarbonate

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solution. The product thus separated was filtered and recrystallized from suitable solvents.

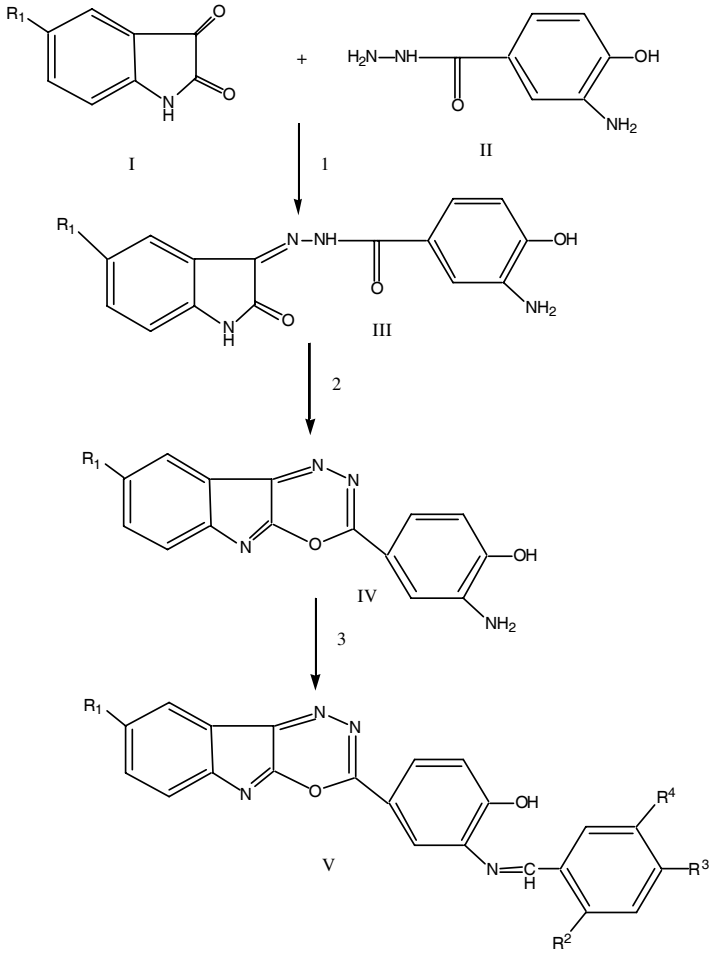
2-[(benzalamino-4-hydroxybenzyl) (1, 3, 4)-oxadiazino (6, 5-b)]indole (V):

Each of the 2-Amino-4-([1,3,4]oxadiazino[6,5-b]indole-3-yl)-phenol (IV, 0.01 mol) was hated with an aromatic aldehyde (benzaldehyde, *p*-Chloro benzaldehyde, Salicylaldehyde, Anisaldehyde, Veratraldehyde, *p*-Dimethylamino benzaldehyde and Vanilaldehyde) in ethanol (20ml) and few drops of acetic acid, heated under reflux on water bath for 3 hours. The solvent was removed to the possible extent by distillation under reduced pressure. The product thus obtained was filtered, washed with water and purified by recrystallization from suitable solvent (Table 1)

For example, 2-Amino-4-([1, 3, 4) oxadiazino [6, 5-b] indole-3-yl)-phenol (IV) was condensed with benzaldehyde to get a single product. This on purification by recrystallization from methanol and DMF (1:1) has resulted in yellow solid, m.p. 286-288^{0}C. It was characterized as 2-(Benzylidene-amino)-4-(1-oxa-3, 4, 9-triaza-fluoren-2-yl)-phenol. Its IR spectrum (in KBr) showed characteristics absorption bands (in cm^{-1}) at 1610 (C=N), 1100 (C-O-C). PMR spectrum (in DMSO-d_6) showed characteristic signals (in δ ppm) at 12.5 (s, 1H, -OH), 7.1 -8.9 (m, 11H, Ar-H).The mass spectrum of the compound showed its molecular ion peak (M^+) at m/z 367. It exhibited the fragmentation pattern characteristic of the compound.

Antimicrobial Activity

The antibacterial activity of the test compounds was assayed against *Bacillus subtilis, Staphylococcus aureus* (gram – positive) and *Escherichia coli* and *Proteus vulgaris*(gram – negative) by CUP-plate method[8]. The antifungal activity of test compounds was determined against *A. niger, C. verticulata, F. oxysporum and A. flavus* by the cup-plate method[9]. The results are presented in Table III.



1. Ethanol 2. Con. H_2SO_4 3. Aromatic aldehyde/Ethanol/few drops of Acetic acid

R1 = H, Br, NO_2 R^2 = H, OH R^3= H, OH, OCH_3, $N(CH_3)_2$ R^4= H, OCH_3

SCHEME - 1

Table I
Data on physical And Analytical of New (1,3,4)oxadiazino-[5,6-b]indole (V)derivatives

Compound	Substituents				Molecular formula	M.P. (^{0}C)	R_f Value	Yield (%)
	R^1	R^2	R^3	R^4				
V(1)	H	H	H	H	$C_{22}H_{14}N_4O_2$	286-288	0.570	83
V(2)	H	H	Cl	H	$C_{22}H_{13}N_4O_2Cl$	295-297	0.541	88
V(3)	H	OH	H	H	$C_{22}H_{14}N_4O_3$	297-299	0.501	85
V(4)	H	H	OCH_3	H	$C_{23}H_{16}N_4O_3$	288-290	0.620	86
V(5)	H	H	OCH_3	OCH_3	$C_{24}H_{18}N_4O_4$	285-287	0.661	90
V(6)	H	H	$N(CH_3)_2$	H	$C_{24}H_{19}N_5O_2$	>300	0.724	87
V(7)	H	H	OH	OCH_3	$C_{23}H_{16}N_4O_4$	272-274	0.586	87
V(8)	Br	H	H	H	$C_{22}H_{13}N_4O_2Br$	280-282	0.728	88
V(9)	Br	H	Cl	H	$C_{22}H_{12}N_4O_2ClBr$	294-296	0.654	89
V(10)	Br	OH	H	H	$C_{22}H_{13}N_4O_3Br$	300-302	0.589	87
V(11)	Br	H	OCH_3	H	$C_{23}H_{15}N_4O_3Br$	290-292	0.694	85
V(12)	Br	H	OCH_3	OCH_3	$C_{24}H_{17}N_4O_4Br$	286-288	0.792	88
V(13)	Br	H	$N(CH_3)_2$	H	$C_{24}H_{18}N_5O_2Br$	240-242	0.929	82
V(14)	Br	H	OH	OCH_3	$C_{23}H_{15}N_4O_4Br$	280-282	0.832	79
V(15)	NO_2	H	H	H	$C_{22}H_{13}N_5O_4$	249-251	0.690	81
V(16)	NO_2	H	Cl	H	$C_{22}H_{12}N_5O_4Cl$	260-262	0.669	83
V(17)	NO_2	OH	H	H	$C_{22}H_{13}N_5O_5$	280-282	0.578	84
V(18)	NO_2	H	OCH_3	H	$C_{23}H_{15}N_5O_5$	256-258	0.720	79
V(19)	NO_2	H	OCH_3	OCH_3	$C_{24}H_{17}N_5O_6$	269-271	0.836	86
V(20)	NO_2	H	$N(CH_3)_2$	H	$C_{24}H_{18}N_6O_4$	280-282	0.939	82
V(21)	NO_2	H	OH	OCH_3	$C_{23}H_{15}N_5O_6$	243-245	0.879	88

Table II
Analytical data of New (1,3,4)oxadiazino-[5,6-b]indole(V)derivatives

Compound No	Calculated						Found					
	C%	H%	N%	O%	Cl%	Br%	C%	H%	N%	O%	Cl%	Br%
V(1)	72.12	3.85	15.29	8.73	-	-	72.26	3.87	15.32	8.76	-	-
V(2)	65.92	3.27	13.98	7.98	8.85	-	65.97	3.29	13.99	7.99	8.88	-
V(3)	69.10	3.69	14.65	12.55	-	-	69.26	3.76	14.69	12.58	-	-
V(4)	69.69	4.07	14.13	12.11	-	-	69.73	4.13	14.16	12.13	-	-
V(5)	67.60	4.25	13.14	15.01	-	-	67.74	4.28	13.18	15.10	-	-
V(6)	70.40	4.68	17.10	7.82	-	-	70.63	4.72	17.13	7.84	-	-
V(7)	66.99	3.91	1359	15.52	-	-	66.93	3.76	13.63	15.58	-	-
V(8)	59.34	2.94	12.58	7.19	-	17.95	59.90	2.94	12.58	7.19	-	17.95
V(9)	55.08	2.52	11.68	6.67	7.39	16.66	55.19	2.90	11.80	6.89	7.90	16.89
V(10)	57.28	2.84	12.15	10.41	-	17.32	57.30	2.58	12.56	10.91	-	17.80
V(11)	58.12	3.18	11.79	10.10	-	16.81	58.45	3.59	11.39	10.70		16.90
V(12)	57.04	3.39	11.09	12.66	-	15.81	57.90	3.29	11.70	12.90		15.99
V(13)	59.03	3.72	14.34	6.55	-	16.36	59.30	3.80	14.50	6.70	-	16.60
V(14)	56.23	3.08	11.40	13.03	-	16.26	56.12	3.50	11.67	13.56		16.57
V(15)	64.23	3.19	17.02	15.56	-	-	64.56	3.80	17.90	15.87	-	-
V(16)	59.27	2.71	15.71	14.36	7.95	-	59.59	2.90	15.90	14.80	7.89	-
V(17)	61.83	3.07	16.39	18.72	-	-	61.90	3.59	16.98	18.80	-	-
V(18)	62.58	3.43	15.87	18.12	-	-	62.98	3.79	15.90	18.90		
V(19)	61.15	3.63	14.86	20.36	-	-	61.49	3.90	14.80	20.98		
V(20)	63.43	3.99	18.49	14.08	-	-	63.80	4.30	18.90	14.40	-	-
V(21)	60.13	3.73	15.24	20.90	-	-	60.178	3.98	15.50	20.98	-	-

Table III

Antimicrobial Activity of New (1,3,4)oxadiazino-[5,6-b]indole(V) derivatives

Compound No.	Antibacterial Activity (Zone of inhibition in mm)				Antifungal Activity (Zone of inhibition in mm)			
	B. Subtilis	*S. Aureus*	*E. coli*	*P. vulgaris*	*A. niger*	*C. verticulata*	*F. oxysporum*	*A. flavus*
V(1)	18	17	16	14	19	16	12	10
V(2)	14	15	14	12	15	13	10	08
V(3)	14	16	13	13	16	15	09	09
V(4)	13	17	13	11	14	15	08	09
V(5)	14	14	14	12	12	13	11	10
V(6)	17	13	15	13	13	12	10	07
V(7)	15	14	14	11	14	13	11	06
V(8)	16	15	15	13	13	14	08	09
V(9)	19	18	23	16	17	16	13	11
V(10)	17	15	19	15	14	14	06	10
V(11)	14	16	20	14	15	11	12	09
V(12)	15	15	18	15	15	12	13	10
V(13)	16	17	19	12	16	13	12	08
V(14)	14	15	18	13	12	14	09	09
V(15)	17	12	13	11	16	14	07	10
V(16)	15	11	14	10	15	13	06	09
V(17)	16	12	13	11	17	12	10	04
V(18)	15	13	12	13	14	13	08	09
V(19)	16	11	14	12	14	12	10	03
V(20)	17	14	13	11	18	14	07	09
V(21)	15	14	11	13	14	15	10	07
Ampicillin (10 µg/cup)	22	20	18	17	-	-	-	-
Clotrimazole (10 µg/cup)	-	-	-	-	21	22	23	15

*Concentration of Test Compound:100 µg/cup

Results and Discussion

The title compounds were characterized by their physical, analytical and spectral data. The details of the compounds have been given in the experimental section.

The antibacterial data of 2-[(benzalamine-4-hydroxybenzyl) (1,3,4)-oxadiazino[6,5-b]]indole (V) indicate that these compounds exhibited a marginal antibacterial activity, interestingly, against all the fours strains of bacterial and almost to the same extent.

The compounds exhibit antifungal activity against all the four strains fungi employed but of course with a degree of variation. These compounds were found to be relatively more effective against *A. niger* and *C. verticulata.*

Acknowledgements

The authors are thankful to the Prof.Dr.B.Agaiah, Principal, SRR.College of Pharmaceutical Sciences for providing necessary facilities to carry out this Research Work

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In- vivo anti-inflammatory activity of ethanolic extract of *Butea frondosa* in albino rats

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ABSTRACT

In the indigenous system of medicine many plants have been found to be useful in the treatment of inflammation. *Butea frondosa* is traditionally used in treatment of inflammation. Ethanolic extract of bark of *B. frondosa* and its isolated compound were subjected to preliminary screening for anti-inflammatory activity in albino rats. The ethanolic extract and isolated compound exhibited significant anti-inflammatory activity comparable to the standard drug Diclofenac sodium against carrageenan induced rat paw edema method. Among these, isolated compound showed maximum anti-inflammatory activity at very low dose than ethanolic extract.

Key words: *Butea frondosa,* inflammation, carrageenan, Diclofenac sodium.

Introduction

Inflammatory diseases are very common throughout the world and pose a great problem to modern society due to its crippling effect, resulting at times in complete invalidity. The problem is further heightened due to lack of any specific treatment. In spite of the discovery of NSAIDS and the emergence of several such newer agents, the search for better anti-inflammatory drugs is continued because these agents have many known side effects as well as very high potency and none of them are suitable for prolonged use. Several laboratories in India are actively engaged in anti-inflammatory drug research from indigenous plants as these are cheap, easily available and have minimum side effects and some plants have shown significant results [1].

Butea frondosa belongs to family *Leguminoceae* is known as flame of the forest. Traditionally poultice of flower and solution of its gum are utilized to reduce inflammation [2]. *B. frondosa* root and leaf extract possess ocular anti-inflammatory activity; the flowers have been reported to exhibit anti-inflammatory and analgesic activity [3, 4]. The root extract possess significant concentration dependent lens protective activity [5]. The literature survey revealed the anti-inflammatory activity in the various parts of *B. frondosa*. However, no work has so far been reported on the anti-inflammatory activity of bark and isolated compound of this plant in the literature. So it was thought worthwhile to investigate anti-inflammatory activity of bark extract and its isolated compound.

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Materials and Methods

Plant material:

Bark of *Butea frondosa* was collected from Mandsaur (MP) and was authenticated by plant taxonomist Dr. C. Rajasekharan. A voucher specimen has been deposited in the herbarium of institute (Pharm/1052). Bark was separated from the wood and used for extraction. The collected material was washed thoroughly in water, chopped, air dried for a week at 35-40^{o}C and pulverized in electric grinder. The powder obtained was defatted with petroleum ether and successively extracted with alcohol. The extract was concentrated under reduced pressure and dried. The *B. frondosa* bark extract (EBE) was column chromatographed over Silica gel using gradient elution with Butanol: Acetic acid: Water; afforded compound-1.

Phytochemical studies:

The EBE was evaluated chemically by performing the qualitative chemical tests and thin layer chromatography [6]. The extract gave positive qualitative tests for Alkaloids, Carbohydrates, Glycosides and Flavonoids. Compound-1 (amorphous brownish yellow powder) found to contain a spot corresponding to literature Rf value of butein.

Experimental animals:

Healthy albino rats of either sex (Wistar strain) weighing 100-160g were used in present study. The animals had free access to food and water and were maintained under controlled temperature (27±2^{0}C) and 12

h: 12 h light and dark cycle. Initial body weight of each animal was recorded.

Acute toxicity studies:

EBE at different doses (50-2000mg/kg) were administered orally to normal rats. During the first four hours after the drug administration, the animals were observed for gross behavioral changes if any for 7 days. The parameters such as hyperactivity, grooming, convulsions, sedation, hypothermia, mortality was observed. No mortality observed with oral administration of all the extracts even at the highest dose (2000mg/kg). Institutional Animal Ethics Committee (IAEC) had approved the experimental protocol and care of animals was taken as per the guidelines of CPCSEA, Department of animal welfare, Government of India.

Test for Anti-inflammatory Activity:

The EBE and compound-1 were tested for anti-inflammatory activity by carrageenan induced rat paw edema method [7, 8]. Healthy albino rats of either sex, weighing 100-160g were selected and provided standard rat feed and water *ad libitum*. Before the experiment, food was withdrawn overnight but adequate water was given to the rats. The animals were divided into seven groups of 6 animals each .The first group (Control group) received acacia (5%, 10ml/kg). The second group received Diclofenac sodium (5mg/kg, positive control). The third, fourth and fifth group received EBE at 200, 300 and 400mg/kg body weight respectively. The sixth and seventh group received Compound-1 at 20 and 40mg/kg body weight respectively. All the drugs were given orally half an hour before the administration of carrageenan suspension. Acute inflammation was produced by the sub-plantar administration of 0.1ml of 1% Carrageenan in normal saline in the left hind paw of the rats. The paw volume was measured at 0, 1, 3 and 5 hrs with the help of plethysmometer. The average paw swelling in the groups of EBE and compound-1 treated rats were compared with control group and the standard group and percent change in edema was calculated**.**

Statistical Analysis:

Results were subjected to statistical analysis by ANOVA and results were expressed as mean ± SEM.

Result and Discussion

Ethanolic *Butea frondosa* bark extract (EBE) and compound-1 showed a significant ($p<0.01$) inhibition of carrageenan induced rat paw edema and the results are presented in Table1and Figure1. The EBE showed 40.81%, 47.38% and 57.61% edema inhibition after 3rd hour at 200, 300 and 400mg/kg dose respectively. Compound-1 showed 47.49% and 62.12% edema inhibition after 3rd hour at 10 and 20mg/kg dose respectively. Maximum activity was found at 3.0 hrs intervals with each dose. Anti-inflammatory activity of EBE and compound-1 is comparable to that of Diclofenac sodium at dose 400mg/kg and 30mg/kg respectively at 3 hrs and duration of action found to be almost same as the standard drug.

The inflammation induced by carrageenan is biphasic in nature. The initial phase of edema has been attributed to the release of histamine and serotonin; the edema maintained during the plateau phase, attributed to kinin like substances and the second accelerating phase of swelling is attributed to the release of prostaglandins [9, 10]. Since the EBE and compound-1 inhibited the carrageenan-induced edema that involves release of histamine and serotonin in the first phase; hence the inhibitory effect of the extract and compound-1 could be partly due to inhibition of mast cell mediator release.

It can be concluded that the Ethanolic *Butea frondosa* bark extract (EBE) and compound-1 possess significant anti-inflammatory activity. Dose dependent study of EBE and compound-1 showed that both had slow onset of action as compared to Diclofenac sodium and the anti-inflammatory effect increased with increase in the dose.

However more elaborate work is required to establish the efficacy of EBE and compound-1 as potent anti-inflammatory drug. Further detailed experimental work is necessary to identify the other active principles present in the EBE and mechanism of action responsible for its anti-inflammatory activity.

Acknowledgements

Authors are deeply grateful to the Department of Pharmaceutical Sciences, HNB Garhwal University, Srinagar (Garhwal) Uttarakhand, SGRR Institute of Technology & Science, Patel Nagar, Dehradun (India) and Department of Pharmacognosy, B. R. N. College of Pharmacy, Mandsaur (MP) for research facilities.

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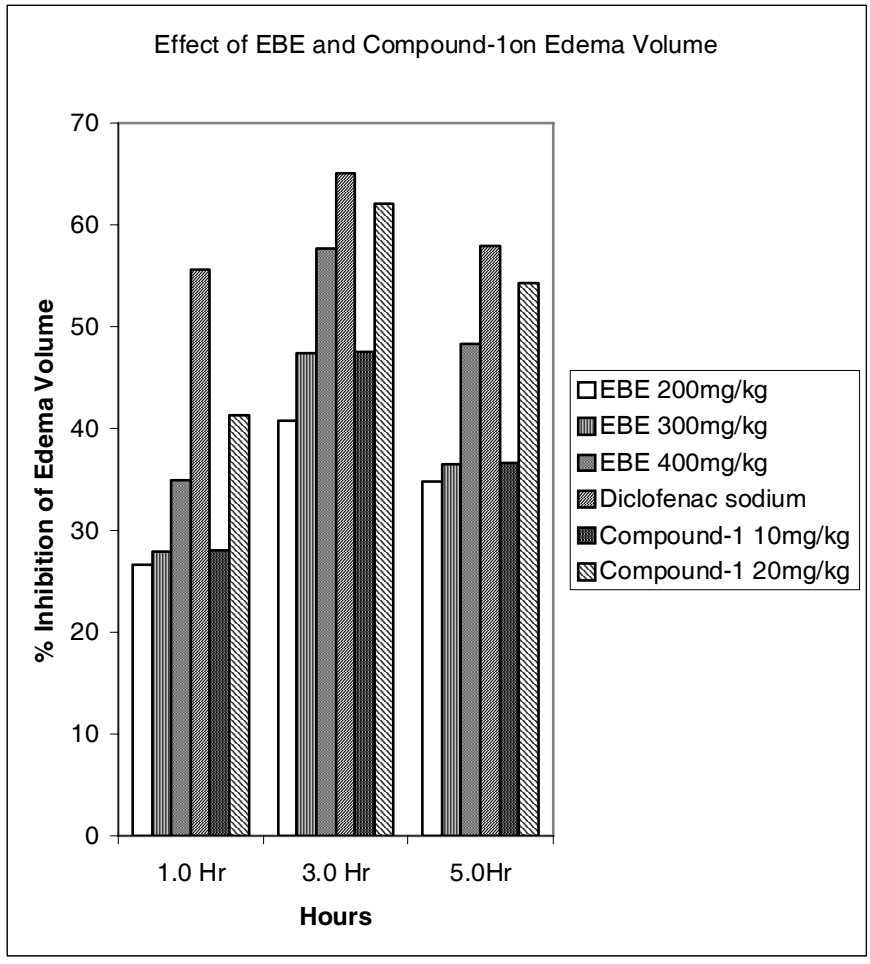
Table 1

Percent Protection Comparison of Alcoholic Bark Extract of *B. frondosa* (EBE) and Compound-1

Treatment	Edema Volume					
	1.00 hr		3.00 hr		5.00 hr	
	(ml)	% Inhibition	(ml)	% Inhibition	(ml)	% Inhibition
Group I (Control)	0.68±0.03	-	0.71±0.04	-	0.69±0.05	-
Group II (Diclofenac sodium, 5mg/kg)	0.30±0.06	55.59	0.25±0.03	65.00**	0.29±0.03	58.00*
Group III(EBE 200mg/kg)	0.50±0.06	26.66	0.42±0.03	40.81*	0.45±0.05	34.78
Group IV(EBE 300mg/kg)	0.49±0.03	28.00	0.37±0.02	47.38*	0.43±0.06	36.54*
Group V(EBE 400mg/kg)	0.44±0.04	34.94	0.31±0.01	57.61*	0.36±0.03	48.29*
Group VI(Compound-1, 20mg/kg)	0.49±0.02	28.15	0.37±0.04	47.49*	0.44±0.02	36.58*
Group VII(Compound-2, 30mg/kg)	0.40±0.01	41.25	0.26±0.02	62.12**	0.31±0.02	54.23*

** Significant at P< 0.001, values are expressed as ± SEM

* Significant at P< 0.01.



Figre-1

Metal contents in the soils of tea garden belt of Sonitpur District, Assam, India

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ABSTRACT

A study has been presented on metal contents in the soils of tea garden belt of Sonitpur district, Assam with focus on iron, copper, manganese, zinc, calcium and magnesium. A total of forty four soil samples collected from inside and outskirts of tea gardens have been studied separately. Normal distribution statistic and reliability analysis have been employed to find out the distribution pattern, localisation of data, and other related information. Statistical observations show all the elements under investigation exhibit an asymmetric dis-tribution with a long asymmetric tail on the right of the median. Diûerences between mean and median in each case, high standard deviation and positive kurtosis indicate that the distribution of metals in the soils of the study area is widely of normal. Soils of the area in general contain high iron and manganese and deficient in copper, zinc, calcium and magnesium. Thus, the intrinsic soil quality in the area is not encouraging because of either low or high nutrient status in soils.

Key Words: Soil quality, Acidity, Quartile, Correlation.

Introduction

Soil is an essential natural resource with which agriculture meets fundamental human needs. Soil is a critically important component of the earth's biosphere, functioning not only in the production of food and fiber but also in ecosystem's function and the maintenance of local, regional and global environmental quality. This illustrates the significance of monitoring of soil quality. Soil monitoring programmes are of considerable interest in India now. The all India soil testing programme was originally planned for the development of sixteen soil testing laboratories in collaboration with the United States Agency for International Development (U.S.A.I.D) and to which eight more were added later [1]. The basic objective of soil testing in India is to give farmers a service leading to better and more economic use of fertilizers and better soil management practices for increasing agricultural production. There are a number of approaches for interpretation of soil data to assess soil quality but as yet there is no international consensus [2-4]. We also do not know how far a soil may deviate from a baseline before this elicits a response (trigger point). Classical quality control theory suggests that once a sample point exceeds a defined number of standard deviations from the mean, then this forms an outlier. An outlier is an observation whose value is distant from the values of the majority of observations but is not necessa-rily due to incorrect measurements. Alternatively, they represent the samples where maximum intervention may be required. Examination of the previous data can establish whether this is an aberrant point or a long-term trend. But unfortunately there is no previously published data available for metal content of soil in Sonitpur district, Assam to compare with. Recently Dutta et al. surveyed soil fertility status with respect to available N, P, K and %C in this area [5]. As further data is obtained it would be possible to establish ranges within which soil quality values should fall.

Metal contents in the soils of tea garden belt of Sonitpur district, Assam with focus on iron, copper, manganese, zinc, calcium and magnesium have been presented in this communication. The implications presented are based on statistical analyses of the raw data. Normal distribution analysis (NDA) and reliability analysis (Correlation Matrix) are used for interpretation of data. Correlation between different parameters in specific environmental conditions has been shown to be useful when such correlation exists and determination of few parameters would sufficient to give some idea about the quality of the soil in the area. The primary objective of this study is to present a statistically meaningful soil quality data-base of the area.

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Materials and Methodology

Sampling methodology:

Forty four soil samples were collected in and around the eleven selected privately owned small tea gardens of Sonitpur district, Assam in November, 2007 where no appropriate chemical testing of soils are done on a regular basis (Table 1). Samples were collected by adopting simple random sampling technique by maintaining a distance of about 50 meters between two samples. A "V" shaped cut of 0 to 6-inch depth at random locations was made in each sampling sites and one inch of soil on either side of pit was scraped and collected in polythene bags. Quartering technique was adopted to reduce the size of the sample to the required mass. The field collected soil samples after assigning identification number were air-dried in oven set at 100 F (38^0C) for 12 hours. The air-dried sample was crushed by hand using a pestle and mortar and analyzed for pH, Fe, Cu, Mn, Zn, Ca and Mg by selecting standard procedures [6].

Table 1
Area wise sample collection summary

Area	Number of tea gardens	Number of samples (Identification No)	Sources	
			Inside Tea garden	Outside Tea garden
Gohpur Biswanath Chariali	7	28 (A-G)	14	14
	4	16 (J-M)	8	8
Total	**11**	**44**	**22**	**22**

Statistical Analysis:

Sample data were subjected to statistical treatment using normal or Gaussian distribution statistic and correlation analysis. Correlation analysis measures the closeness of the relationship between chosen independent and dependent variables. If the correlation coefficient is nearer to +1 or -1, it shows the probability of linear relationship between the variables. Details of these may be found in standard books on statistics and software packages [7]. Some more statistical estimates derived from the normal distribution were also made in the present study for analysing soil quality data and have been shown below:

Sample variance (r^2): Sample variance is given as the square of the standard deviation (r).

Kurtosis: Kurtosis is an indicator of the relative sharpness or fatness of the peak compared to normal distribution. Positive kurtosis indicates a sharp distribution while negative kurtosis indicates a flat one.

Skewness: A measure of the asymmetry of a distribution. The normal distribution is symmetric, and has a skewness value of zero. A distribution with a significant positive skewness has a long right tail. A distribution with a significant negative skewness has a long left tail. As a rough guide, a skewness value more than twice its standard error is taken to indicate a departure from symmetry.

Percentile (P_i): Percentile at 25%, 50%, 75% were calculated. Pi at 25% is called first quartile, at 50% second quartile and at 75% third quartile. Pi is also known as the cumulative probability function which lies in the range $0< Pi < 1$ for i =1, ..., n.

Results

To look into the trend and distribution patterns of metals in soils of tea garden belt of Sonitpur district data were exposed to several statistical treatments, which have been briefly discussed in Materials and Methodology section. A conventional descriptive statistics based on normal distribution has been shown in Table 4 and 6 with regard to pH, Fe, Cu, Mn, Zn, Ca and Mg in soils.

Table: 2
Soil quality parameters inside tea gardens

Sample No.	pH	Fe (ppm)	Cu (ppm)	Mn (ppm)	Zn (ppm)	Ca (meq/100g)	Mg (meq/100g)
A1	3.48	45.2	0.536	7.21	0.176	0.2	1.1
A2	3.82	46.1	0.697	7.63	0.185	0.4	1.0
B1	4.83	72.4	4.012	42.13	1.541	0.2	0.4
B2	4.95	70.8	4.023	45.16	1.736	0.4	1.3
C1	3.48	71.2	0.781	48.31	1.921	0.3	0.9
C2	3.90	67.4	0.730	15.39	0.248	0.2	1.3
D1	4.33	69.4	0.762	4.82	0.673	0.2	1.0
D2	4.72	66.9	0.743	4.09	0.480	0.6	1.4
E1	4.70	109.2	1.092	21.24	0.985	0.5	1.3
E2	4.50	120.1	1.119	23.23	1.080	0.3	0.9
F1	5.00	54.0	0.652	3.23	0.217	0.8	0.9
F2	4.50	53.1	0.599	3.54	0.177	0.6	0.8
G1	4.50	57.3	0.876	1.926	0.413	0.2	0.4
G2	4.80	54.2	0.686	1.838	0.220	0.3	0.8
J1	4.50	69.8	0.785	1.761	0.203	0.4	0.4
J2	4.78	70.3	0.748	1.621	0.197	0.6	0.7
K1	4.80	69.5	0.752	1.767	0.747	0.3	0.4
K2	5.10	65.4	2.190	9.861	0.781	0.8	0.8
L1	5.20	63.3	2.376	10.412	0.740	0.2	0.2
L2	5.50	60.8	2.270	1.963	0.691	0.6	0.3
M1	4.87	48.8	2.092	2.808	0.872	0.2	0.2
M2	5.10	44.6	1.981	2.408	0.786	0.6	0.5

Table: 3

Soil quality parameters outside tea gardens

Sample No.	pH	Fe (ppm)	Cu (ppm)	Mn (ppm)	Zn (ppm)	Ca (meq/100g)	Mg (meq/100g)
A11	4.85	38.6	0.682	5.943	0.098	0.3	0.50
A12	4.92	39.1	0.798	6.273	0.112	0.4	0.62
B11	4.96	70.4	2.126	4.13	0.783	0.4	1.84
B12	5.02	71.1	3.011	4.21	0.871	0.8	1.90
C11	5.23	61.4	0.773	11.52	0.219	0.2	0.83
C12	5.28	61.27	0.650	12.23	0.228	0.2	1.01
D11	4.24	66.9	0.783	4.915	0.102	0.1	1.32
D12	4.72	68.7	0.842	5.012	0.112	0.2	1.68
E11	4.70	24.3	0.56	1.647	0.119	0.7	2.27
E12	4.50	23.6	0.52	1.587	0.098	0.4	1.94
F11	5.00	51.4	0.741	2.274	0.171	0.2	1.70
F12	4.96	58.6	0.733	10.95	0.837	0.2	1.40
G11	4.70	46.9	0.705	2.456	0.172	0.2	2.81
G12	5.10	49.2	0.876	3.462	0.214	0.4	3.72
J11	40.10	100.1	0.954	2.028	0.352	0.2	1.62
J12	5.10	110.3	1.210	2.425	0.421	0.4	2.40
K11	4.90	71.4	0.791	1.697	0.692	0.4	3.50
K12	4.50	70.6	0.691	1.425	0.498	0.3	2.60
L11	4.99	66.0	0.614	2.595	0.616	1.0	1.70
L12	5.20	69.2	0.873	2.791	0.752	1.2	1.94
M11	4.90	54.3	0.486	3.420	0.230	0.4	0.30
M12	5.50	58.7	0.594	3.691	0.321	0.6	0.40

Discussion

pH: Hydrogen ion concentration acts like a catalyst in maintaining plant growth by the mechanism of optimum plant nutrients uptake. Soils in the range 5.6 to 6.0 are moderately acidic and below 5.5 are strongly acidic in nature [8]. Copper and zinc are most readily available from pH 5 to 7; and iron and manganese are abundant below pH 5, but moderately available from pH 5 to 7. The factors like constant addition of chemicals to the soil along with excessive rainfall results in severe acidity build up in the soil system and affect the nutrient uptake of the tea plantation. In general, since the soil is biodynamic, variation in pH may either result in nonavailability of nutrients in the available form to the plant or excessive availability of a particular nutrient, resulting in unbalanced growth of the plant or starvation of a particular nutrient. Significant negative skewness value for pH inside and out side tea gardens indicates a departure from symmetry.

Iron (Fe): Iron is the fourth most common element in soil, comprising 5% of the earth's crust. The Fe in soil is usually found in the soluble cationic form (Fe^{2+}). All the soil samples of the study area have high iron contents and cross the critical limit as suggested by Olson and Carlson [9]. A very broad third quartile in case of iron represents a long asymmetric tail on the right of the median. This analysis clearly reveals that soils of Sonitpur district are alarmingly contaminated with iron.

Copper (Cu): Solubility of copper in soil is highly pH dependent. Soils hold copper most securely at pH 7-8, appreciably less securely at pH 6 and as the soil acidity increases further, copper is held very loosely [10]. That is the reason why copper content of soil is poor in the area. Correlation between copper and pH is found to be significant

Table: 4

Normal distribution statistic for pH, Fe, Cu, Mn, Zn, Ca, and Mg inside tea gardens

Statistics	pH	Fe	Cu	Mn	Zn	Ca	Mg
Mean	4.6073	65.9000	1.3865	11.9248	0.6850	0.4045	0.7727
Std. Error of Mean	0.11333	3.91629	0.22381	3.17583	0.11061	0.04288	0.08063
Median	4.7500	66.15000	0.78300	4.4550	0.6820	0.3571	0.8167
Mode	4.50	44.60	0.54	1.62	0.180	0.20	0.40
Std. Deviation	0.53158	18.36905	1.04978	14.89597	0.51881	0.20113	0.37819
Variance	0.28257	337.42190	1.10203	221.88987	0.26916	0.04045	0.14303
Skewness	-0.845	1.722	1.588	1.658	1.067	0.627	0.041
Std. Error of Skewness	0.491	0.491	0.491	0.491	0.491	0.491	0.491
Kurtosis	0.327	3.675	1.794	1.573	0.519	-0.807	-1.150
Std. Error of Kurtosis	0.953	0.953	0.953	0.953	0.953	0.953	0.953
Range	2.02	75.50	3.49	46.69	1.75	0.60	1.20
P 25%	4.3980	54.0000	0.7300	1.9630	0.2170	0.2364	0.4200
P 50%	4.7500	66.1500	0.7830	4.4550	0.6820	0.3571	0.8167
P 75%	4.9500	70.3000	2.0920	15.3900	0.8720	0.5667	1.0333

Table: 5

Correlation matrix of different soil quality parameters inside the tea gardens

pH	Fe	Cu	Mn	Zn	Ca	Mg	
pH	1.0000						
Fe	0.0362	1.0000					
Cu	0.5151	0.0462	1.0000				
Mn	-0.2396	0.4230	0.5285	1.0000			
Zn	0.0645	0.4081	0.6452	0.8612	1.0000		
Ca	0.4406	-0.0648	-0.0530	-0.2473	-0.1981	1.0000	
Mg	-0.4521	0.2666	-0.2575	0.2857	0.0001	0.1582	1.0000

Table: 6

Normal distribution statistic for pH, Fe, Cu, Mn, Zn, Ca, and Mg outside tea gardens

Statistics	pH	Fe	Cu	Mn	Zn	Ca	Mg
Mean	4.8805	60.5486	0.9097	4.3946	0.3645	0.4182	1.7273
Std. Error of Mean	0.07095	4.35076	0.12278	0.68882	0.05797	0.06017	0.19775
Median	4.9333	61.3350	0.7570	3.4410	0.2290	0.3444	1.7000
Mode	4.50	23.60	0.49	1.43	0.10	0.20	1.70
Std. Deviation	0.33277	20.40687	0.57590	3.23086	0.27189	0.28223	0.92753
Variance	0.11074	416.44052	0.33166	10.43846	0.07393	007965	0.86031
Skewness	-0.653	0.416	2.963	1.548	0.761	1.516	0.411
Std. Error of Skewness	0.491	0.491	0.491	0.491	0.491	0.491	0.491
Kurtosis	0.591	1.171	9.083	1.501	-0.978	1.949	-0.066
Std. Error of Kurtosis	0.953	0.953	0.953	0.953	0.953	0.953	0.953
Range	1.40	86.70	2.53	10.81	0.77	1.10	3.42
P 25%	4.7067	49.2000	0.6500	2.2740	0.1190	0.2222	1.0100
P 50%	4.9400	61.3350	0.7570	3.4410	0.2290	0.4000	1.7000
P 75%	5.0733	70.4000	0.8730	5.0120	0.6160	0.5500	2.2700

Table: 7

Correlation matrix of different soil quality parameters outside the tea gardens

pH	Fe	Cu	Mn	Zn	Ca	Mg	
pH	1.0000						
Fe	-0.0340	1.0000					
Cu	0.1000	0.3510	1.0000				
Mn	0.3750	-0.0570	-0.0310	1.0000			
Zn	0.2460	0.4600	0.5720	-0.0170	1.0000		
Ca	0.3620	-0.0200	0.2170	-0.3420	0.4750	1.0000	
Mg	-0.1730	0.1400	0.1420	-0.4900	0.2460	0.0880	1.0000

at the 0.05 level inside the tea gardens. Positive kurtosis and skewness in each set of samples is indicative of the asymmetric nature of copper distribution.

Manganese (Mn): Soil manganese exists in equilibrium between plants available Mn^{2+} and unavailable Mn^{3+} forms. Plants take up manganese as Mn^{2+} from the soil solution. It is fairly mobile in the soil and can be leached, particularly on acid soils. The low pH of soils in the area favour reduction of Mn^{3+} to Mn^{2+}. Thus, at low pH levels (less than 5.5), manganese becomes very soluble and manganese toxicity occurs. Toxicity is usually associated with other acid soil infertility problems such as deficiencies of calcium and magnesium. Correlation analysis of data also establishes this relation in the study area. High third percentile and positive kurtosis value inside the tea gardens is indicative of sharp non uniform distribution of manganese in the soil.

Zinc (Zn): Zinc availability in soils is at its minimum at pH values between 5.5-7.0 and the situation becomes more complex when pH increases to more than 7.0 [11]. Zn in soil solution exists as Zn^{2+}. As a positive ion, it is quite immobile in soil. At lower pH, the yield is reduced. The experimental results after comparing with the critical ratings given for zinc show that the soils inside as well as outside tea gardens of Sonitpur district, Assam have low zinc content [12]. Zinc content of the area shares a significant correlation with copper at the 0.01 level. Outside the tea gardens correlation between zinc and iron is also significant at the 0.05 level. Asymmetric nature of Zn distribution is also apparent from the width of the third quartile which is much greater than the second quartile inside as well as outside tea gardens.

Calcium and Magnesium (Ca & Mg): Ca and Mg are classified as secondary nutrients. They are secondary only in the probability of deficiencies and are taken up by plants in quantities similar to phosphorus. We have measured the amounts of exchangeable Ca and Mg since this is the plant-available form. Calcium and magnesium deficiency symptoms can be rather vague since the situation often is accompanied by a low soil pH. The acidic soils of the study area limit the availability of calcium and magnesium to the plant. It is also observed that Ca and Mg have a significant correlation with pH at the 0.05 level inside the tea gardens. The distributions for both appear to be asym-metric and flat with the common feature of third quartile being wider than the second and negative kurtosis values.

Conclusion

A comprehensive statistical analysis of metal con-tents in soils of tea garden belt of Sonitpur district, Assam has been carried out with special reference to Fe, Cu, Mn, Zn, Ca and Mg. Statistical observations show that all these elements exhibit an asymmetric distribution with a long asymmetric tail on the right of the median. The width of the third quartile was consistently found to be more than the second quartile for each element. Differences between mean and median in each case, high standard deviation and positive kurtosis in most of the cases indicate that the distribution of metals in the soils of the study area is widely of normal. Wide data range in each case indicates the presence of extreme values in the form of outliers, which are likely to bias the normal distribution statistic. Thus, the inherent quality of soils in and around the tea gardens of Sonitpur district, Assam is low because of either low or high nutrient status in soils.

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Acid -stable amylase production from newly isolated *Aspergillus sp.*by submerged fermentation

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ABSTRACT

Extracellular acid stable amylase production characteristics were investigated in newly isolated fungal strain, *Aspergillus sp.* The imperative role of physiological factors, nutritional components and influence of ion concentration on biomass and enzyme production was investigated. The enzyme production in this fungal isolate is observed to be partly constitutive and partly inducible by catalytic product, maltose. The amylase enzyme production is regulated by the pH of the medium, phosphate ion concentration and type of carbon and nitrogen source in addition to physiological growth factors such as temperature and agitation in this fungal strain.

Key words: *Aspergillus sp.*, Acid-stable amylase; enzyme production.

Introduction

Enzymatic impact on modern biotechnological processes over chemical catalysts is substantially increasing day-by day mainly due to their requirement to catalyze the reaction in relatively mild conditions of temperature and pH, exquisite selectivity both in reactant and product stereochemistry in addition to their renewable and biodegradable nature. Hence enzymes made a significant contribution in industrialization of the planet. Among different extra-cellular enzymes, amylases are the most important biocatalysts due to their wide application spectrum in many industrial sectors such as textile, food, brewing and starch based industries[1]. In addition, recent trend in use of amylases in clinical, medical and analytical fields enhanced the amylase industrial share up to 25% of total enzyme market globally. Among all enzymes, microbial amylases have a panoramic use in biotechnological processes.

Though several amylase producing bio-systems including bacterial, fungal, plant and animal are characterized for their importance in production of this enzyme, only *Aspergillus* genus acquired great significance in the present day biotechnology. This is mainly due to its harmlessness characteristic nature, its potential to produce and secrete extracellularly in relatively large quantities in addition to their simple and common growth requirement [1]. However, present enzyme limitations in utilization for specific applications in upcoming sectors, restricted commercial production of amylase enzyme by existing microbes and the requirement of catalytic specific nature of enzyme in the vast improving modern biotechnology warrants the need to find more suitable candidates to improve upon the process economics to replace old generation benign methods to isolate microbial strain producing amylase suitable to modern industrial application, in spite of widely available amylase producing organisms. This can be achieved by isolating new enzyme producing strains and their optimization for growth as well as amylase production capabilities.

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Materials and Methods

Microorganism and culture conditions

A fungal isolate, of *Aspergillus* sp. from onion was used in the present study. The microorganism was grown regularly in a medium consisting of soluble starch – 2.0%, beef extract - 0.3 % and peptone - 0.5% with pH 5.0 and incubated at 33^{O}C.The agar slants were sub-cultured at monthly regular intervals. The spore suspension of 10^8 spores per ml was prepared under sterilized conditions from fully-grown agar plates for uniform inoculum and stored at 4^{O}C till further use.

Fermentation conditions

For production of acid-stable amylase by this isolate, 100 ml medium consisting of above media components

was prepared in 250-ml Erlenmeyer flasks and autoclaved at 121^{O}C for 15 minutes at 15 lbs. Under aseptic conditions, these flasks were inoculated with 2 ml of the above spore suspension and incubated at predetermined temperature and rpm in an orbital shaker. Whenever required the medium was supplemented with KH_2PO_4 (0.2%), K_2HPO_4 (0.2%), $MgSO_4.7H_2O$ (0.1%), different carbon sources (soluble starch, potato starch, dextrin, maltose, glucose, fructose, sucrose, xylose, glycerol, lactose and lactic acid (2% (w/v) each)) and different nitrogen sources (corn steep liquor, soyabean meal, peptone, ammonium chloride, sodium nitrate, ammonium nitrate, urea, aspartic acid, glycine, and glutamic acid (0.5%)) before autoclaving. The cell free broth of these flasks collected at regular predetermined time intervals was used as enzyme source.

Measurement of mycelial biomass, protein content and specific activity

The fungal biomass was estimated by collecting the mycelial biomass after filtration, drying at 60^{O}C overnight in hot air oven and weighing. The enzyme content in the cell free broths was quantified in terms of protein content, which was measured using Lowry's method [2]. The specific activity was calculated by estimating the ratio of enzyme activity and protein content.

Estimation of amylase activity

The amylase activity was measured in cell free broths collected at regular and predetermined incubation times according to Bernfeld [3] by estimating the maltose produced during starch hydrolysis using modified dinitrosalicylic acid as a coupling reagent.

One unit of the enzyme is defined as the amount of enzyme capable of producing 1µM of reducing sugar (as maltose) from 1% soluble starch as substrate in 1min at 40^{O}C and p^{H} 4.8. The enzyme activity was calculated using the following formula [4].

Amylase activity(u ml^{-1})=Maltose (mg)/360.31 X 10^{6}

Results and Discussion

The role of medium pH on amylase production by Aspergillus sp.

pH of the growth medium reported to influence the growth of the organism and metabolic product formation and its secretion in case of extracellular products[1]. In general, amylase production by most of the microbial organisms under submerged fermentation conditions is observed to be optimum in the pH range between 6.0 and 7.0. However, production of amylase enzyme and growth of the organism also observed in different pH conditions depending on the organism's physiology. The results indicated that, pH of the medium influences the growth of this fungal strain and enzyme production level and both these production patterns were parabolic in nature (Fig 1). This *Aspergillus* strain showed maximum biomass (4% dry mycelial weight) and enzyme production (2208 units/ml) at pH 5.0 after 48 hours of incubation time indicating this strain is acidophilic in nature and the amylase produced is acid-stable. This data suggest that, this *Aspergillus* sp. and produced enzyme are more tolerant to acidic pH rather than pH increase towards alkalinity. Further analysis of the data revealed that the amylase production was increasing with increase in incubation time irrespective of the growth medium pH indicating the production of amylase enzyme in this fungal strain is growth associated.

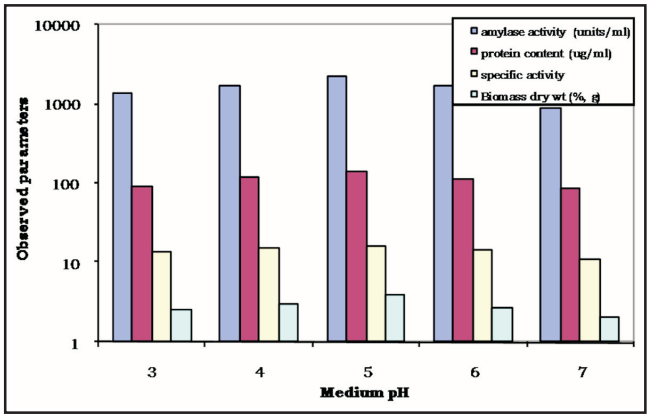

Fig 1: Role of medium pH on various parameter production by isolated *Aspergillus* sp.

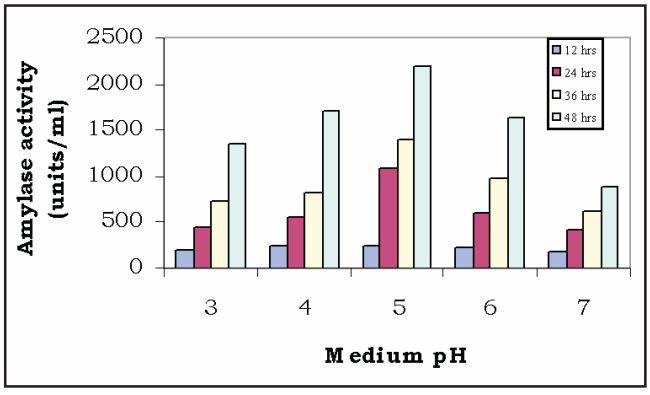

Fig 2: Influence of medium pH on acid-stable amylase production by isolated *Aspergillus* sp.

Effect of incubation temperature on acid-stable amylase production

The influence of temperature on amylase enzyme production is related to the microorganism growth [5]. Incubation temperature dependent variation in amylase production was also reported in several microbial species [1]. To investigate the same on growth and enzyme production by the isolated *Aspergillus* sp., this strain was incubated at different temperatures (30 to 35^{O}C) in the incubator shaker at 200 rpm for 48 hours. The biocatalyst production and mycelial growth were monitored at 48 hours of incubation (Fig 3 & 4). The results indicated that the incubation temperature regulated amylase production. Maximum amylase production was noticed at a temperature of 33^{O}C.

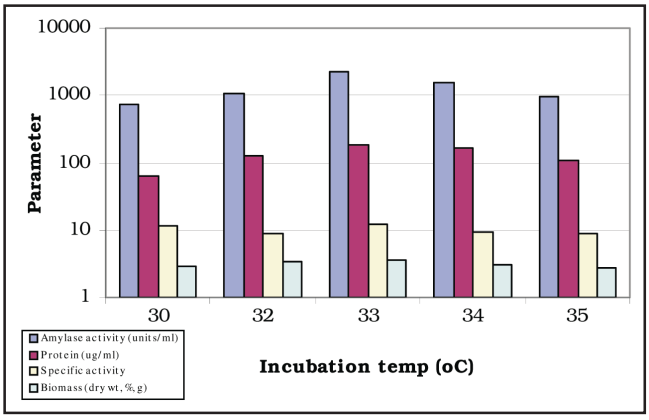

Fig 3: Effect of temperature on production of various parameters by isolated *Aspergillus* sp.

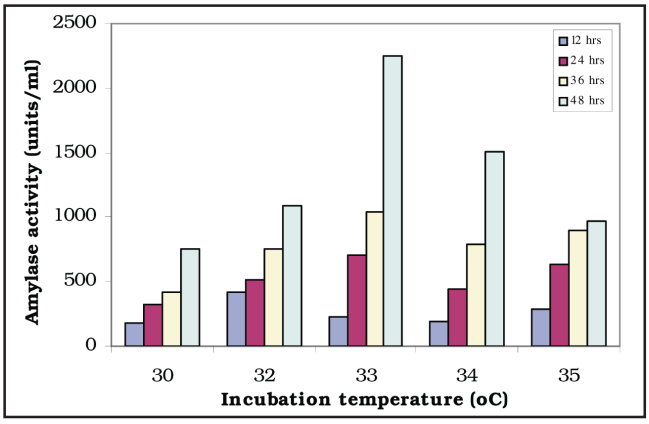

Fig 4:Role of temperature on amylase production by isolated *Aspergillus* sp.

Influence of initial inoculum level on acid-stable amylase production

Quantum of initial biomass controls the kinetics of growth and several biological metabolic functions leading to the overall biomass and extracellular product production [6]. To study the same, experiments were planned with increasing inoculum concentration from 1.0 to 2.5% and the acid-stable amylase activity was monitored during growth phase of this fungal strain up to 48 hours and subsequently the protein content and mycelial biomass (Fig 5 & 6) were estimated.

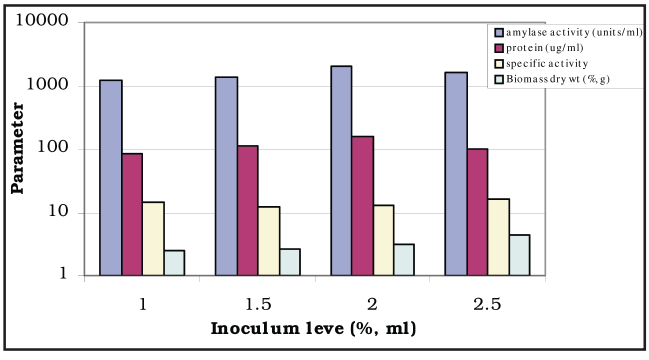

Fig.5: Inoculum level effect on various parameters production by isolated *Aspergillus* sp.

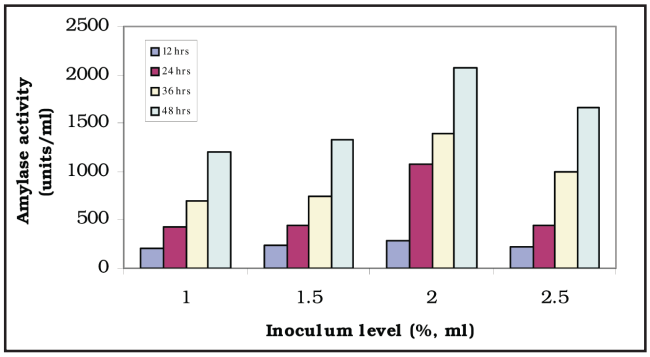

Fig.6: Inoculum leve effect on amylase production by isolated *Aspergillus* sp.

The results indicated that, amylase production kinetics varied with variation in initial inoculum level (Fig 5). Maximum enzyme production was observed at 48 hours of incubation time in 2.0% initial inoculum supplemented conditions.

Role of agitation on acid-stable amylase production

Understanding of mass transfer of substrates, products, nutrients and gases among the system components is one of the important parameter to be considered for optimal biotechnological production of any metabolite or product and exploitation of microbial capability under fermentation process. This is basically achieved in various laboratory scale experiments by agitating the culture components in controlled environmental set up. To evaluate the impact of agitation on acid-stable amylase production by this fungal strain, experiments were carried out in different agitation conditions and the growth of amylase production kinetics were followed. The data indicates that the production of amylase and biomass were influenced by agitation of fermentation broth (Fig 7 & 8). Maximum enzyme activity was observed in 200 rpm grown culture broth at 48 hours of incubation. Change in agitation speed in either side of 200 rpm resulted in reduction of enzyme activity.

Phosphate ion role in acid-stable amylase production

Phosphate ion plays an important regulatory role in the synthesis of primary and secondary metabolites in microorganisms in addition to its as buffering action in the growth medium [1,7,8]. However, phosphate role is found

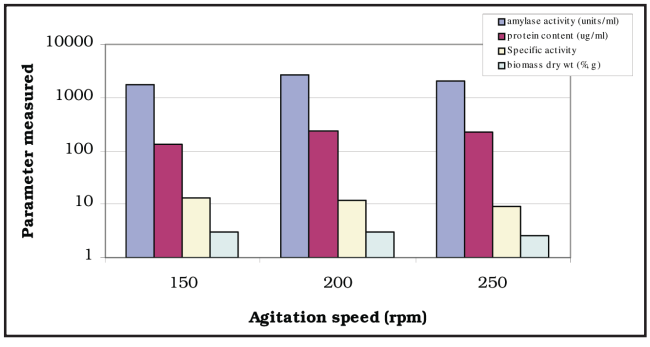

Fig 7: Effect of agitation on growth and acid stable amylase production by *Aspergillus* sp.

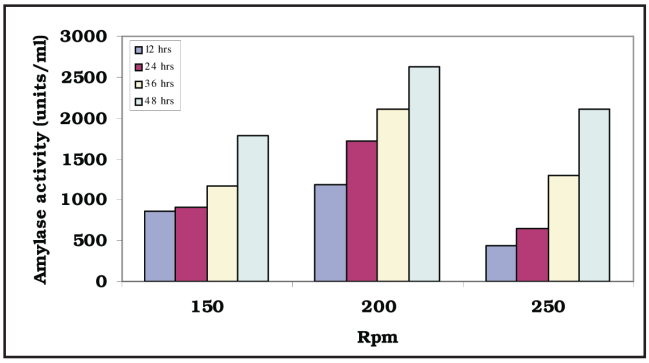

Fig 8: Effect of agitation on acid stable amylase production by *Aspergillus* sp.

to be organism specific as it has been reported that phosphate ions enhances the growth of organism and amylase production in *Aspergillus oryzae*[9] while ion mediated repression of amylase production was noticed in *Bacillus amyloliquefaciens* [10,11]. The amylase production studies in presence of phosphate ions (KH_2PO_4; 0.2%) in this fungal strain showed improved production (30%) over control indicating the positive regulation of amylase production (Fig 9 & 10). This phosphate ion mediated regulation of amylase in this organism was associated at the enzyme synthesis level rather than at activity level. This was confirmed with the observed increase in enzyme quantity in terms of protein content in the cell free culture broth. Such phosphate ion concentration mediated regulation

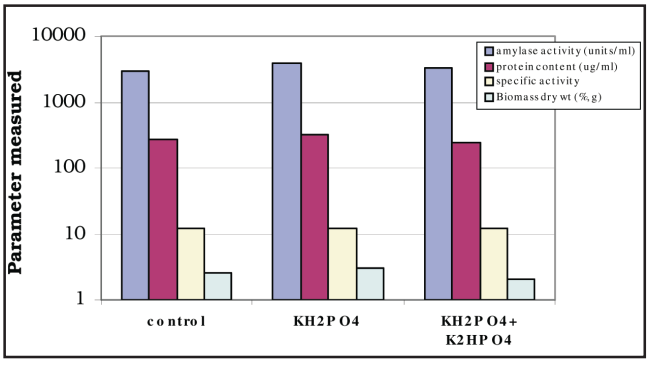

Fig 9: Influence of inorganic phosphate on growth and enzyme production by *Aspergillus* sp.

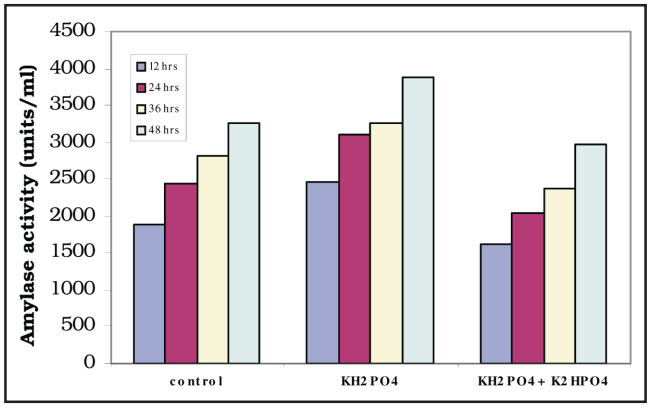

Fig 10: Role of inorganic phosphate on acid stable amylase production by *Aspergillus* sp.

of biomass and amylase production was also reported in *Bacillus amyloliquefaciens*[11].

Influence of magnesium ion on acid-stable amylase production

Magnesium is one of the typical and most abundant divalent cations present inside the cells and known to act as coordinator to phosphoryl oxygen atom. In addition, it serves as cofactor for numerous enzymes, helps in maintainance of the pH balance and ion transport and metabolism [12]. Magnesium ion mediated regulation of amylase production was also reported in *Aspergillus oryzae* EI 212 [13]. While the enzyme synthesis noticed to be increasing in the presence of magnesium ions in *Bacillus* sp. CRP strain [14]. To understand the role of magnesium ions on amylase production in isolated *Aspergillus* sp. was investigated by growing the fungal strain in magnesium ion ($MgSO_4.7H_2O$; 0.1%) supplemented growth medium. Analysis of amylase activity indicated marginal increase (10%) in enzyme activity in magnesium supplemented conditions over control indicating magnesium ions regulatory role in amylase production in this isolated strain.

Role of carbon substrates on acid-stable amylase production

Amylase is generally an inducible enzyme and most of the induction studies reported that this enzyme is induced in the presence of starch or its hydrolytic product, maltose [15-18]. The results are reported in Fig 11 & 12. It is clear from the data that carbon source mediated influence on amylase production. Maltose and soluble starch were most effective in the induction of acid-stable amylase production in this strain among all investigated carbon sources. These results are in accordance with the observations noticed in amylase producing *Aspergillus oryzae* (NRC 401013) *A.oryzae* DSM 63303 and *Aspergillus nidulans* [17]. The amylase production in maltose supplemented conditions was found to be 3141 units/ml, which was 50% higher with maltose as inducing substrate than soluble starch indicating the importance of catalytic product in elevating the enzyme induction process. The observed variation in

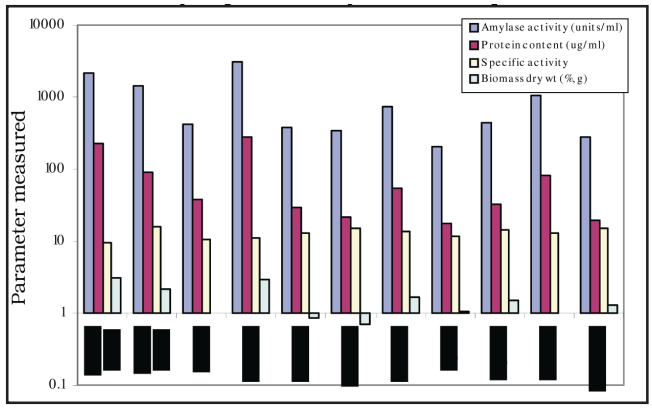

Fig 11: Different carbon sources effect on growth and enzyme production by *Aspergillus* sp.

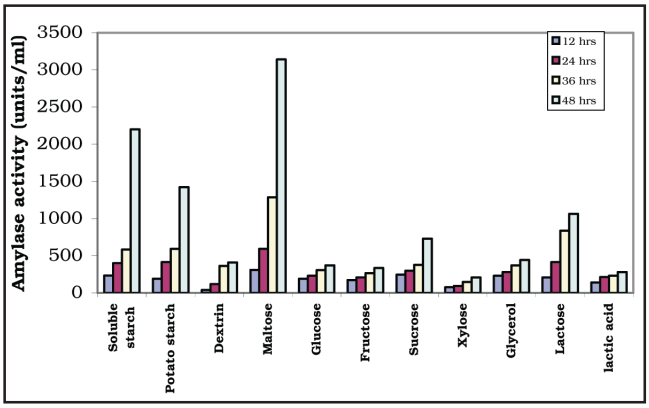

Fig 12: Role of different carbon sources on acid stable amylase production by *Aspergillus* sp.

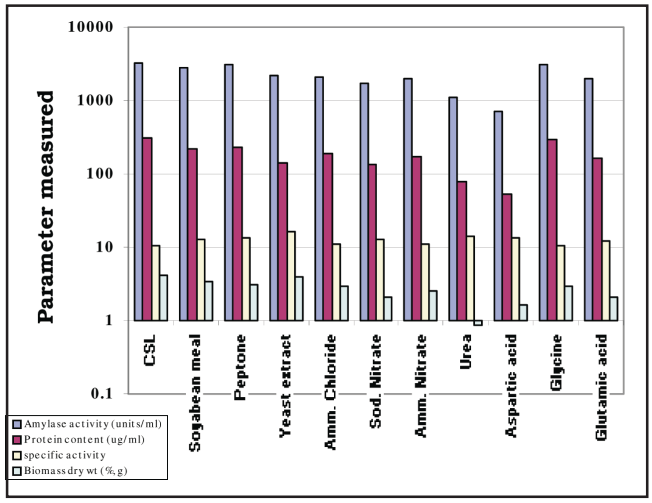

Fig 13: Influence of different N-sources on growth and enzyme production by *Aspergillus* sp.

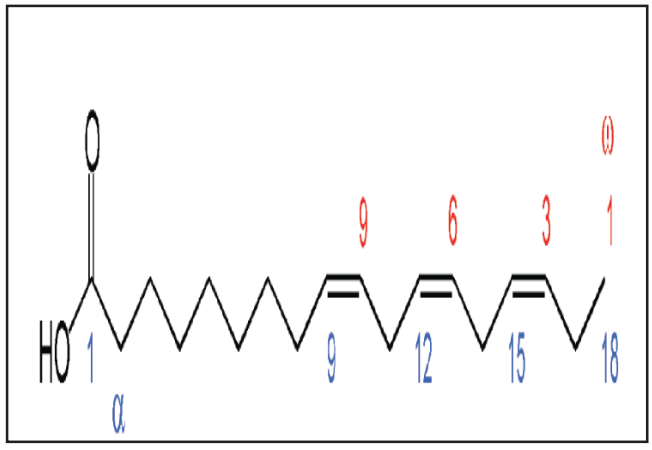

Fig 14: Influence of different N-sources on acid stable amylase production by *Aspergillus* sp.

amylase activity in soluble starch (2200 units/ml) and potato starch (1422 units/ml) grown cultures may be attributed to the role of the structure of the substrate in induction of amylase enzyme during the growth of this fungus.

Influence of nitrogen sources on acid stable amylase production

Nitrogen source mediated amylase enzyme production was observed in various amylase-producing microorganisms [18]. The imperative role of different nitrogen sources on acid stable amylase production by this fungal strain was investigated by supplementing the selected nitrogen source (0.5%) to the medium in place of yeast extract. The data revealed that among all studied nitrogen sources, complex nitrogen sources supported amylase as well as biomass production (Fig 13 & 14). In the present investigation, peptone and CSL supported more than 40% enzyme productivity over yeast extract as nitrogen source indicating acid stable amylase production in this fungal strain is influenced by type of complex nitrogen compound.

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Development and Validation of an RP-HPLC Method for the Determination of Levetiracetam in Tablet Dosage Forms

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ABSTRACT

A rapid and reproducible reverse phase high performance liquid chromatographic method has been developed for the determination of Levetiracetam from its dosage forms. The separation was effected on a C_{18} Bondapak column (250 mm x 4.6 mm; 5ì) using a mobile phase consisting of methanol and water (70:30 v/v) at a flow rate of 1ml/min. Zaleplon was used as an internal standard and the detection of the drug was made at 222 nm. The retention time of the drug was found to be 3.24. The method produced linear responses in the concentration range of 1-60 ìg/ml. The proposed method was validated as per the ICH and USP guidelines. The method is accurate and precise and found to be suitable for the quantitative analysis of the drug in its pure form and in tablet dosage forms.

Key words: Levetiracetam, Determination, Tablets, HPLC.

Introduction

Levetiracetam is a soluble ethyl analogue of the piracetam, a nootropic agent. It is chemically, á- ethyl -2-oxo- (- á s) 1-pyrrolidine acetamide. It is a novel anti-epileptic drug approved by the U.S. Food and Drug Administration in the treatment of seizures [1]. The major metabolic pathway of levetiracetam is the enzymatic hydrolysis of the acetamido group, which is not liver cytochrome P-450 dependent. About 91% of the dose is excreted via the renal route [2]. So far, a few HPLC methods were reported for the estimation of levetiracetam in human plasma, in therapeutic drug monitoring studies and in bulk drugs and dosage forms [1-8]. The authors now propose a rapid, sensitive and validated HPLC method for the estimation of levetiracetam in tablet dosage forms.

Materials and Methods

Chromatographic conditions

A Waters HPLC instrument equipped with a 510 solvent delivery pump, a Bondapak C_{18} column, a Waters 486 tunable absorbance detector and a 25 µl Hamilton syringe was employed in this study. HPLC grade water (E.Merck) and methanol (Qualigens) were used for preparing the mobile phase. A mobile phase consisting of a mixture of methanol and water (70:30 v/v) was used in this study. The mixture was filtered through a 0.45µ membrane filter and then sonicated with a UCB 30-ultrasonicator before use.

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Drug samples

The working standard sample of levetiracetam and the commercial sample of, Torleva tablets used in the study were gifted by Torrent Pharmaceuticals Ltd.

Preparation of standard solution of levetiracetam

About 50 mg of Levetiracetam was weighed accurately and transferred into a volumetric flask and dissolved in 25ml of 70:30 v/v methanol and water. The contents were sonicated well for about 15 min and the volume made up with the mobile phase to get a 1 mg/ml solution. This solution was suitably diluted further with the mobile phase to get different dilutions in the range of 1-60 ìg/ml.

Preparation of the internal standard solution

The stock solution of the internal standard was prepared by dissolving 50 mg of Zaleplon in 50 ml of the mobile phase in a volumetric flask and sonicating for 15 min. From this stock solution (1 mg/ml), a suitable aliquot was taken and diluted with the mobile phases to get a 100 ìg/ml solution.

Estimation of the drug and Calibration curve

The quantitative estimation of the drug was carried out by the internal standard method. Prior to injection of the drug solution, the column was equilibrated for at least 30 min. with the mobile phase. Different dilutions of levetiracetam ranging from 1 -60 ìg/ml were from the standard solution in different volumetric flasks to which a suitable aliquot of internal standard solution was also added uniformly, to get a final concentration of 50 ìg/ml of internal standard in each mixture. The contents of the flask were made up with the mobile phase. These mixtures

were filtered through a 0.45 ì filter before use. Each of these samples was injected five times into the column and the peak area ratios of drug to that of internal standard were calculated. The flow rate of the mobile phase was set at 1 ml/min. and the detection was monitored at 222 nm. The retention times of the drug and internal standard were found to be 3.2 min. and 5.4 min respectively. A calibration curve was plotted by taking the peak area ratios of the drug to the internal standard on the y- axis and concentration on the x-axis as per the data presented in Table 1.

Table 1
Calibration of the proposed HPLC method

Concentration of Levetiracetam (µg/ml)	Mean Peak area ratio (n=6)	% RSD
1	0.0251	0.25
10	0.1682	0.03
20	0.2748	0.98
40	0.5601	0.65
6 0	0.8752	0.28

Estimation of the drug from tablet dosage forms

Twenty tablets of Torleva were weighed and powdered. From this an amount of the powder equivalent to 100 mg of levetiracetam was weighed and dissolved in a small quantity of the mobile phase and sonicated for 15 min. The volume was then made up to 100 ml with the mobile phase to get a 1 mg/ml solution. This stock solution was diluted with the mobile phase so as to obtain dilutions in the range of linearity obtained for the pure drug. An aliquot of the solution of zaleplon was added to all the dilutions to get a concentration of 50 ìg/ml of the internal standard in each solution.

Results and Discussion

The present study was aimed to develop a rapid, precise and accurate HPLC method for the analysis of the levetiracetam from its dosage forms. For this, a binary mixture of methanol and water in 70:30 v/v proportion was found to be suitable to get well defined and resolved peaks free from tailing in the chromatogram. A model chromatogram showing separation of levetiracetam is shown in Fig 1.

The proposed method was validated in terms of linearity, precision and accuracy as per ICH and USP guidelines [9]. Each of the sample solutions was injected 5 times and the same retention times for the drug and the internal standard were observed in all the cases. The ratios of peak area of levetiracetam to that of the internal standard for different concentrations setup as above were calculated and the average value for five such determinations are shown in Table 1. The peak areas of both the drug and the internal standard were reproducible as indicated by low percent RSD. A good linear relationship (r=0.9991) was observed between the concentration of levetiracetam and the respective peak area ratios. The regression curve was constructed by linear regression fitting and it was expressed as y=0.0101x+0.0139 (where y represents the ratios of peak areas of the drug to that of the internal standard and x is the concentration of levetiracetam). The regression characteristics are given in Table 2.

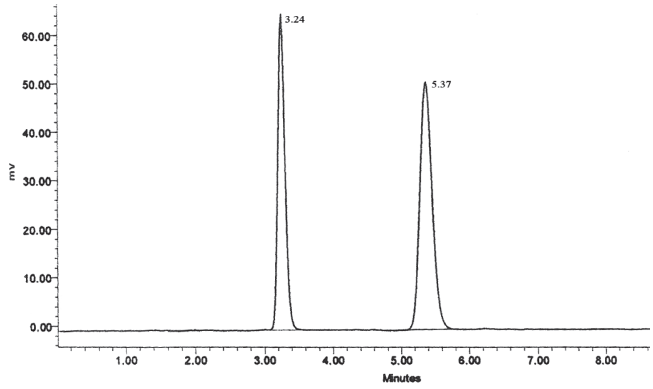

Fig.1: Typical Chromatogram for the determination on Levetiracetam

Table 2
Regression characteristics of the proposed HPLC methods

Parameter	Value
Detection limit (µg/ml)	1-60
Detection Wavelength	222
Relative standard deviation (%) (n=6)	0.2721
% Range of error at 95% confidence limit	0.3710
% Range of error at 99% confidence limit	0.5490
Slope (a)	0.0139
Intercept (b)	0.0101
Correlation coefficient (r)	0.9991

A high correlation coefficient value and y- intercept close to zero indicates the good linearity of the proposed method. Low percent RSD values were observed, when levetiracetam solutions containing 5-15 ìg/ml were analysed by the proposed method for finding out intra-day and inter-day variations (Table 3). This shows that the present HPLC method is highly precise.

Table 3
Precision of the proposed HPLC method

Concentration of levetiracetam (µg/ml)	Observed concentration of levetiracetam (µg/ml)			
	Intra-day		Inter-day	
	Mean (n=5)	% RSD	Mean (n=5)	% RSD
5	4.97	0.55	4.78	0.62
10	10.09	0.91	9.98	0.87
15	14.99	0.24	15.01	1.32

The mean amount of levetiracetam obtained from its tablet dosage form as shown in Table 4 indicates that the proposed method is highly accurate.

Table 4
Recovery of levetiracetam from tablet dosage form

Brand Name of the drug	Labeled amount (mg)	Mean (±s.d.) amount found	% RSD
Torleva	250	250.2± 0.85	0.34

The values of the system suitability parameters studied as per USP guidelines are shown in Table 5. The low values of LOD and LOQ indicate the high sensitivity of the proposed method.

Table 5
System suitability parameters

Parameter	Value
Theoretical plates (n)	5844
Resolution (R)	4.086
HETP	4.775×10^{-3}
Tailing factor (T)	2.5
LOD	0.321
LOQ	1.091

Conclusion

The proposed HPLC method is rapid, sufficiently sensitive and reproducible for the determination of levetiracetam from its tablet dosage forms and thus can be used for the routine quality control analysis with short run times.

Acknowledgement

The authors are thankful to Torrent Pharmaceuticals Ltd for providing the drug samples and dosage forms for the study.

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Comparitative and quantitative analysis of solvent extracted Linseed oil of Indian origin, based on dielectric constant and expressed oil with other varieties

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ABSTRACT

Presence of highest percentage of PUFA's (omega-3 and omega-6) and mono unsaturated fatty acids (omega-9) increased the usage of linseed as well as the linseed oil in diet. Present study is on the lipid composition of the oil extracted from different solvents based on the dielectric constant and the comparitative study of Indian variety collected in the month of January with the other cultivars available in the world shows that the Indian variety contains 30% - 35% of oil content. Lipid composition of the Indian variety contains 9% less of linolenic acid, equal percentage of linoleic acid and 6% more of oleic acid. There is no arachidic acid content in the Indian variety. Commercially available expressed oil has shown the similar composition as that of the solvent or solvent system extracted oils. Hence, usage of commercially available is economical and avoids the expensive extraction procedures.

Key words: PUFA's, Omega-3fatty acid, Omega-6 fatty acid, Omega-9 fatty acid, ±- Linolenic acid, Linoleic acid.

Introduction

Linseed (*Linum Usitatissimum*, Linaceae) is the source of poly unsaturated acids like omega -3, omega-6 and omega-9-fatty acids along with stearic and palmitic acids [1] and [2]. Omega -3- fatty acid is an essential fatty acid in human health which has to be ingested. This fatty acid is also present in soya bean, rape seeds and fish oils. Chemical name *cis*,*cis*,*cis*-9,12,15-Octadecatrienoic acid; 9,12,15-Octadecatrienoic acid; Industrene 120 **(Fig.1)** [2].

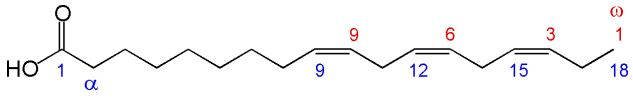

Fig.1: α - Linolenic Acid

Linoleic acid is an EFA taken in the diet and metabolized to gama linolenic acid used by the body to make prostaglandins Prostaglandins are involved in many processes in the body, including regulation of the immune system.It has been suggested that gama linoleic acid supplements are benefited in the people who can not make gama linoleic acid from linoleic acid **(Fig.2)** [2].

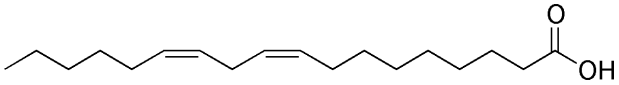

Fig. 2: Linoleic Acid

**Author for Correspondence*

(*cis-cis*-9,12-octadecadienoic acid).

GLA is also present in evening primrose oil, black currant oil, and borage oil.

Oleic acid is a mono unsaturated fatty acid. 9-octadecenoic acid **(Fig.3)**.

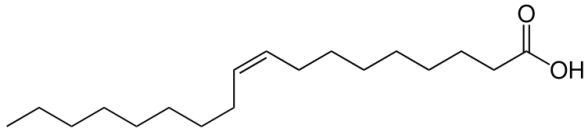

Fig.3:Oleic Acid

PUFA's (omega-3 and omega-6) present in the linseed are effective in reducing the LDL levels in the humans without effecting the HDL levels [11] , [12]. Decrease the total blood cholesterol [9]. Reduces the risk factors of coronary heart diseases (CHD) [10], arthritis [3], anti thrombic, anti inflammatory [4], auto immune diseases [5], anti carcinogenic [8], also in atherosclerosis [7] and suppress the cancer associated cachexia.[6].

Lately, linseed has become so popular as it contains both omega-3 and omega-6 fatty acids. Indian origin linseed contains 30-35% of oil content [1]. Our present study involves the extraction of the linseed oil of Indian origin based on the dielectric constant.

Materials and Methods

Locally available material is collected for the extraction. Solvents(1. n-hexane(2.0), 2.toluene(2.38), 3.diethyl ether (4.34), 4.chloroform(5.5), 5.ethyl alcohol(24.3) and 6.distilled water(78.5) with the increasing polarity from 2.0 to 78.5.

50gms of linseed ground to coarse powder in the mixer and macerated with 150ml of the solvent for 24 hrs. Oil is extracted by decantation followed by filtration and evaporating the solvent at 55 - 60°C on the heating mantle. The residue is re-extracted with the 100ml of the same solvent. Similar procedure is followed for all the other solvents for the extraction of the oil content from the linseed. These oil collected separately from the above solvents subjected to acid hydrolysis (free fatty acids) and esterification (methylated esters of fatty acids(FAME) (samples 1-5).These FAME are passed through sodium sulphate (to remove the moisture content) before gas chromatography analysis for the lipid composition **(Fig 4)**.

Dielectric constant of solvent mixture (n-hexane: ether in the ratio2.3:1) is adjusted to 2.7 as the dielectric constant of linolenic acid is 2.6-2.9. The FAME are collected from the seeds by the above process (sample 6), analyzed for the lipid composition using gas chromatography **(Fig. 6)**.

Commercially available expressed oil in the market is collected and subjected to acid hydrolysis and esterification to get FAME (sample 8), also analyzed for the lipid composition **(Fig. 7)**.

Results and Discussion

Gas Chromatography (GC 6890 with DB225MS) analysis of the samples 1-5 has shown the following percentages oil palmitic, stearic, linoleic and linolenic acids as in **Fig. 4** and **Table 1.**

Lipid composition of the linseed varieties from Poland, Russia, Uruguay, Great Britain, and Canada [2] contains arachidic acid in the range of 0.11 to 1.22 percentages but the Indian variety of linseed has not shown the presence of the arachidic acid on Gas Chromatographic analysis.

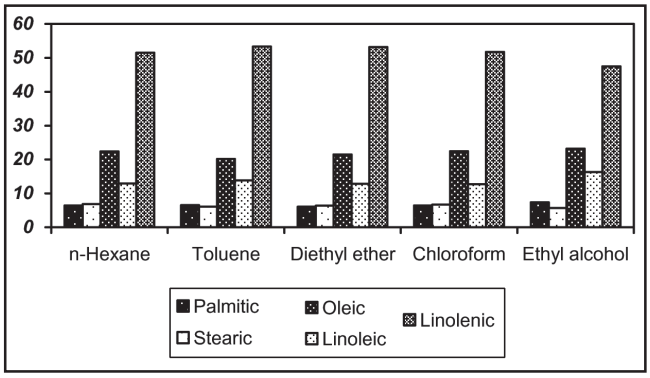

Fig.4: Average lipid composition of the Indian variety of linseed oil extracted from different solvents.

Linseed of Indian variety contains average total PUFA of 65.96% (omega-3(51.432%) and omega-6(14.526%) and omega-9 is 21.94% whereas the other varieties contains 75% of PUFA's (omega-3 (60.5%) and omega-6(14.5) and omega-9 is15.7% (Table 1 and 2) [2].

Gas chromatogram of the oil extracted from the solvent mixture (n-hexane : diethyl ether in 2.3 : 1 ratio) of dielectric constant 2.7 and the commercially available expressed oil shown the following results **(Fig.5)**:

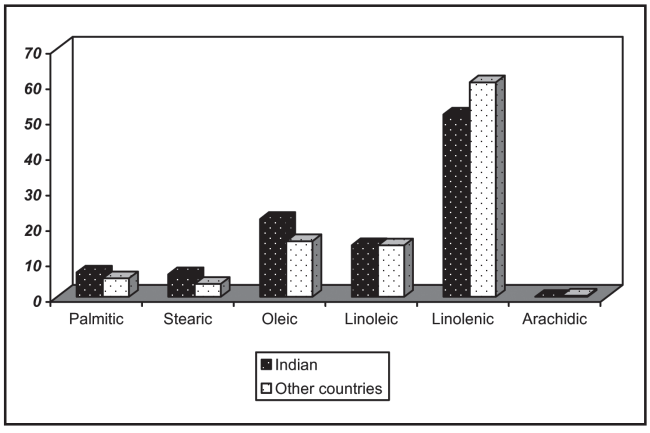

Fig.5: Comparitative analysisof the lipid composition of the linseed of Indian variety to the cultivars of other countries.

Table 1

Lipid composition of the oil extract of Indian variety of linseed with different solvents (samples 1-5).

S.No.	Solvent	Percentage of fatty acids				
		Palmitic	Stearic	Oleic	Linoleic	Linolenic
1	n-hexane(2.0)	6.444	6.8553	22.369	12.858	51.473
2	Toluene(2.38)	6.595	6.075	20.204	13.839	53.287
3	Diethylether (4.34)	6.087	6.394	21.474	12.839	53.207
4	Chloroform(5.5),	6.407	6.697	22.419	12.766	51.711
5	Ethyl alcohol(24.3)	7.334	5.674	23.219	16.285	47.483
	Average :	**6.8705**	**6.33906**	**21.937**	**14.5255**	**51.4322**

Table 2:

Comparision of the lipid composition of Indian variety of linseed oil to cultivars of other countries.

Cultivars	Average percentage of fatty acids					
	Palmitic	**Stearic**	**Oleic**	**Linoleic**	**Linolenic**	**Arachidic**
Indian	6.8705	6.339	21.937	14.525	51.432	Nil
Other countries	5.21	3.65	15.7	14.5	60.5	0.33

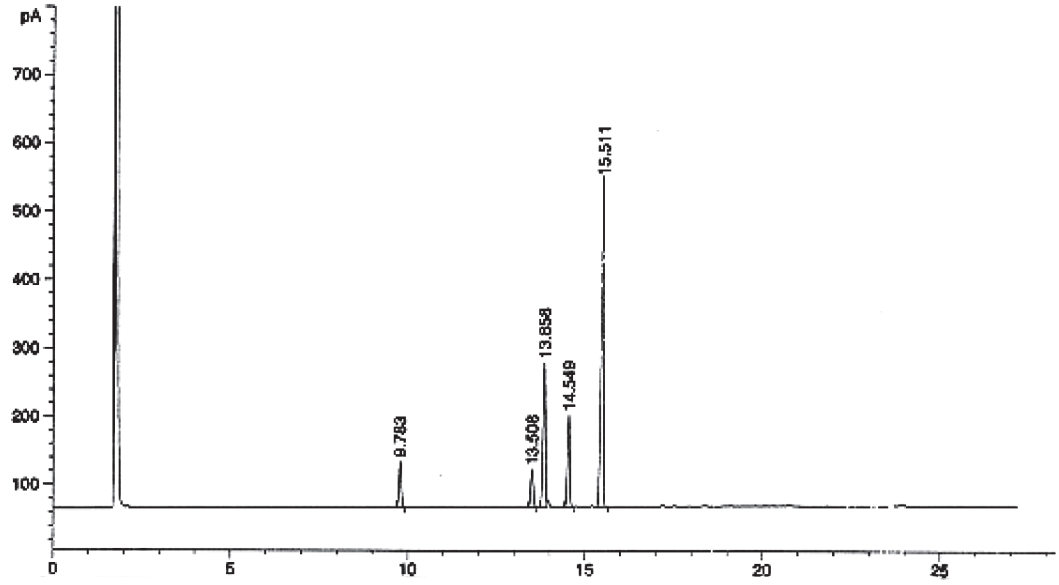

Fig.6: Gas chromatogram of the lipid composition of the Indian variety of linseed extracted from solvent mixture n-hexane + ether (2.3:1).

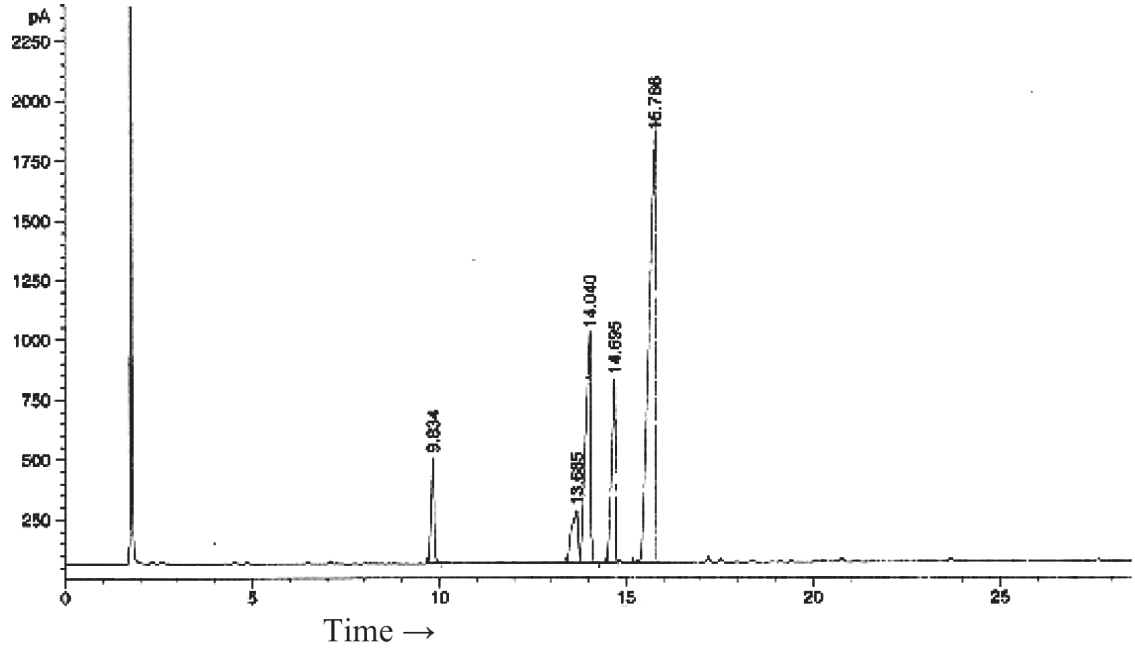

Fig.7: Gas Chromatogram showing the lipid composition of FAME of expressed linseed oil.

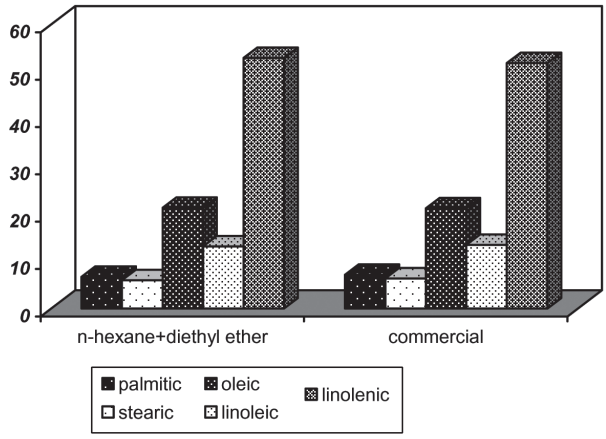

Fig.8: Comparitative lipid composition of the oil obtained from the solvent system of dielectric constant 2.7 and expressed oil.

Gas chromatographic analysis of the oil obtained from the solvent system of dielectric constant 2.7 (n-hexane and ether (2.3:1)) shows almost equal lipid composition as that of the commercially available expressed oil using hydraulic press (Fig.7 and Fig. 8). Hence, commercially available oil is equally rich in omega-3, omega-6 and omega-9 fatty acids, can be used directly. It avoids the expensive procedures of extracting the oil from the seeds using solvents and solvent systems.

Conclusion

Solvent extracted or expressed linseed oil from the commercially available Indian variety of linseed is a rich source of 80.5% PUFA's (±-linolenic and linoleic acids) of the total oil content along with mono unsaturated oil (oleic- 21.94%). Indian variety of linseed oil do not contain arachidic acid when compared with the lipid composition of the other varieties available in the world [2].

Though, linseed oil contains PUFA's in high percentage can not be used as cooking oil as it becomes saturated at high temperatures. It is highly recommended to be used in dressings.

Acknowledgments

One of the authors Sirisha.M would like to thank Principal A.V.Narayana Charya, all my colleagues and the management of the Anwarul Uloom College of Phamacy, Mallepally, Hyderabad 500 001.

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Synthesis, antimicrobial anthelmintic and insecticidal activity of some new 1, 3,4 thiadiazole and thioxo-imidazolidine derivatives

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ABSTRACT

The objective of the present study is to synthesize some new derivatives of 1,3,4 thiadiazole and imidazolidine and to investigate the antimicrobial, anthelmintic and insecticidal activity studies of them.

3-(substituted benzylidene) thiosemicarbazide has been used as a precursor to synthesize some important biologically active heterocycles. Reaction of 1-(substituted benzylidene amino) 1-acetyl-2-thioxo imidazolidine-4-one with acetic anhydride yields imidazolidine derivatives and reaction of 3-(substituted benzylidene) thiosemicarbazide with acetic anhydride gave 1,3,4 thiadiazole derivatives. Several derivatives have been synthesized and screened for their antimicrobial efficacy against *Bacillus* subtilis, *Escherichia* coli, *Staphylococcus* aureus, and *Klebsiella* pneumoniae, Antifungal activity against *Aspergillus* flavus, *Fusarium* oxisporum *Aspergillus* niger and *Trichoderma* viridae, insecticidal Activity against *Periplaneta* americana and anthelmintic activity against *Pheretima* posthuma.

Keywords : 1,3,4 Thiadiazole, tetra hydroimidazole (imidazolidine), antibacterial activity,antifungal activity, insecticidal activity, anthelmintic activity.

Introduction

The cyclisation of suitable linear compounds is one of the most common and popular methods for preparing heterocyclic compounds [1]. In the family of heterocyclic compounds , nitrogen containing heterocycles with a sulfur atom are an important class of compounds in medicinal chemistry [2]. 1,3,4-Thiadiazole and related compounds are of great interest in chemistry owing to their bioactivity of certain plant growth regulating effect as well as antimicrobial activity [3,4].

Antitubercular activities of thiadiazoles linked with aromatic cycles through the methylene oxy group have also been reported and compounds of this type have shown inhibition on both cycloxygenase and 5-lipooxygenase activities [5,6]. Lee and coworkers have synthesized some thiadiazoles having anthelmintic activity [7,8]. More recently, sulfonamide derivatives of 1,3,4-thiadiazoles have been reported to behave as a modulator of anticancer therapies in combination with some cyclotoxic compounds [9-12]. Imidazoles also have great importance in synthetic organic chemistry. Such as Neticonazole is an antifungal used for the treatment of fungal skin infections [13, 14]. Econazole and Abunidazole containing imidazole moiety are antifungal drugs.

Phenyl benzimidazole sulfonic acid is a common sunscreen agent. Burimamide is an antagonist at the H_2 and H_3 histamine receptors. It is largely inactive as an H_2 antagonist at physiological pH [15]. Benzimidazole is an antiparasitic agent and omeprazole is an proton pump inhibitor used in treatment of dyspepsia, peptic ulcer diseases [16]. Imidacloprid have great affinity for insect nitotinergric acetyl choline receptors [17,18].

In the present investigation we have used thiosemicarbazide moiety as a precursor. It represents versatile synthons for various syntheses of nitrogen heterocycles. The thiosemicarbazide moiety provide an opportunity to perform cycloaddition as well as addition cyclization reactions. The products of these cyclization reactions are thiadiazoles, imidazoles, triazole etc. all possess substantial pharmaceutical potential.

The aim of this work is to prepare several derivatives of tetra hydroimidazole moiety and several derivatives of 1,3,4 thiadiazole moiety for their antibacterial, antifungal, anthelmintic and insecticidal activity studies.

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Experimental

All the melting points have been determined in an open capillary and are uncorrected. The reaction were monitored on TLC. The IR spectra have been recorded in KBR pellets on a Shimadzu 8201 PC spectrophotometer (V_{max} in cm^{-1}) ^1H NMR spectra on a Avance II 400 NMR spectrometer (chemical shift in δ ppm, elemental analysis has been carried out on a Carlo Elba 1108 model analyzer.

Preparation of 1-(3'-chloro benzylidene) thiosemicarbazide I(a) :

A mixture of m-chloro benzaldehyde (0.01 mole), thiosemicarbazide (0.01 mole) and 2.5 ml of glacial acetic acid in ethanol (30 ml) was refluxed for 5 hr. on water bath. After the completion of reaction, excess of solvent was removed under reduced pressure. The solid thus obtained was washed with alcohol, then dried and recrystallized from benzene.

Preparation of 3-(3'-chloro benzylidene amino)-2-thioxo imidazolidin-4- One II(a) :

Compound Ia (0.01 mole), ethylchloroacetate (0.01 mole) and fused sodium acetate (0.01 mole) in ethanol was refluxed for 4 hr. then cooled and poured into water.

The resulting solid was filtered off washed with water, dried purified by butanol to give (II). Its purity has been checked by TLC.

Preparation of 3-(3'-chloro benzylidene amino)-1-acetyl-2-thioxo imidazolidin-4-one III(a) :

A solution of IIa (0.01 mole) in acetic anhydride (30 ml) was refluxed for 4 hr. then cooled and poured into ice water. The resulting product was filtered off, washed with water, dried and purified by benzene to give (IIIa). IR V_{max} in cm^{-1} (Ar-H) 2958.9, (C=C) 1606.76, (C=N) 1512.24, (N-N) 833.28 (C-Cl) 968.30, (C=O) 1678.13, (C=S) 1305.85, (C-N) 1255.7;1HNMR ($CDCl_3$) δ ppm, 6.21 (m, unsym, substituted benzene ring); 4.07 (singlet), 3.02 (singlet CH_2), 3.46 (δ, C-H).

Similarly the remaining Imidazolidine derivatives 3(b-1) have been synthesized.

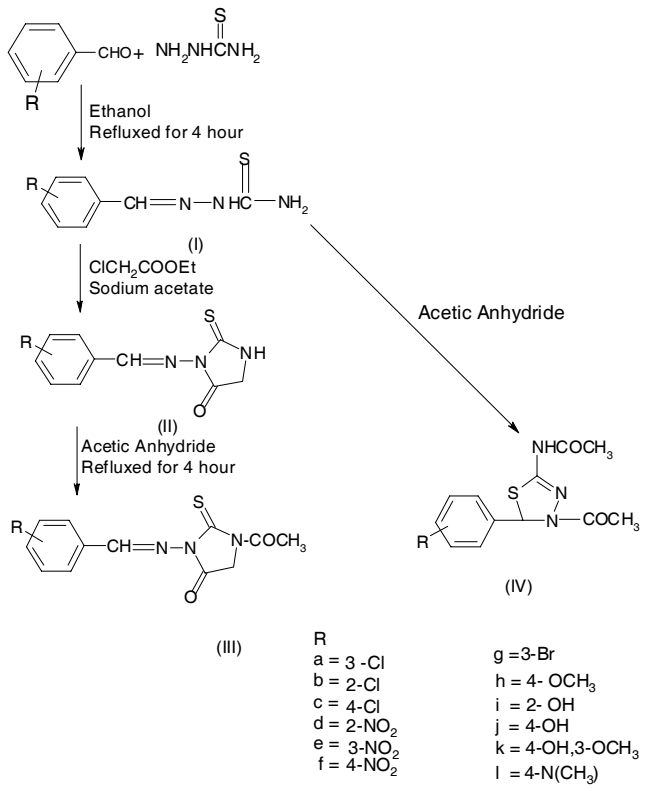
Preparation of N-(4-acetyl-4,5-dihydro-5-(3'-chloro phenyl)-1,3,4-thiadiazol-2-yl) acetamide IV(a) :

Thiosemicarbazone (Ia) (0.05 mole) and acetic anhydride 30 ml was refluxed for 5 hr. The reaction mixture was cooled then poured into ice cold water and kept for 3 hr. The solid thus obtained was filtered and then washed with water, dried and recrystallized from benzene gave the product (IVa).

IR Vmax in cm^{-1} (Ar-H) 3066.92, (C=O) 1766.85, (C=C) 1610.61, (Ar-Cl) 856.42, (N-H) 3215.15, (C=N) 1695.49, (C-N) 1284.63, (N-N) 1033.88, (C-S) 719.47, ^1H NMR ($CDCl_3$) in δ ppm, 7.46 (s, N-H), 4.37 (s, 2 $COCH_3$), 4.03 (s, C-H), 6.43 (m, unsym, chlorosubstituted benzene ring).

Similarly the remaining 1,3,4-thiadiazole 4(b-1) have been synthesized using the same procedure.

Reaction sequence of synthesized Imidazolidine and 1,3,4-thiadiazole Derivatives :



Result and Discussion

The reaction of substituted aromatic aldehyde, thiosemicarbazide and glacial acetic acid in ethanol gave 1-(substituted phenyl benzylidine) thiosemicarbazide I (a-l).

In this step Schiff 's bases are formed further the compound I (a-l) on treatment with ethyl chloro acetate and fused sodium acetate in ethanol gave II (a-l) that refluxed in acetic anhydride has given 3 (substituted phenyl) benzylidene amino)-1-acetyl-2-thioxo imidazolidin-4-one III (a-l).

Compound II (a-l) was refluxed 5 hr. with acetic anhydride and gave N-(4-acetyl-4,5-dihydro-5-(substituted phenyl)-1,3,4-thiadiazole-2yl) acetamide IV (a-l).

Both Imidazolidine and 1,3,4-thiadiazole derivatives have been screened for their antibacterial activity against *Bacillus subtilis, Escherichia coli*, *Klebsiella pneumoniae* and *Saphylococcus aureus* at two different concentration 50 ppm and 100 ppm respectively by filter papers disc plate method and antifungal activity against *Aspergillus niger, Aspergillus flavus*, *Fusarium oxisporum* and *Trichoderma viridae* by filter paper disc plate method at two different concentration (100 ppm and 500 ppm) standard antifungal drug *Griseofulvin* and antibacterial drug *Streptomycin* have also been screened under the similar conditions for comparison.

Table 1
Molecular formula, Mole. weight, Yields and Melting points of the synthesized heterocyclic derivatives IIIa-l and IVa-l.

Compd.	R	Molecular Formula	Mole-cular Weight	Yield (%)	M.P. (ºC)	Elemental Analysis					
						% of Carbon		% of Nitrogen		% of Hydrogen	
						Found	Calcu.	Found	Calcu.	Found	Calcu.
III a	3-Cl	$C_{12}H_{10}ClN_3O_2S$	297.54	63	65	48.71	48.73	14.19	14.21	3.40	3.41
III b	2-Cl	$C_{12}H_{10}ClN_3O_2S$	297.54	62	59	48.70	48.73	14.20	14.21	3.39	3.41
III c	4-Cl	$C_{12}H_{10}ClN_3O_2S$	297.54	64	59	48.69	48.73	14.18	14.21	3.39	3.41
III d	2-No_2	$C_{12}H_{10}N_4O_4S$	306.3	68	68	47.05	47.06	18.27	18.29	3.27	3.29
III e	3-No_2	$C_{12}H_{10}N_4O_4S$	306.3	68	58	47.05	47.06	18.27	18.29	3.28	3.29
III f	4-No_2	$C_{12}H_{10}N_4O_4S$	306.3	69	62	47.04	47.06	18.28	18.29	3.27	3.29
III g	-Br	$C_{12}H_{10}BrN_3O_2S$	340.2	62	69	42.35	42.37	12.34	12.35	2.94	2.96
III h	-4OCH_3	$C_{13}H_{13}N_3O_3S$	291.33	69	82	55.58	55.60	14.40	14.42	4.48	4.50
III i	-2OH	$C_{12}H_{11}N_3O_2S$	277.274	65	68	51.97	51.98	15.13	15.15	3.98	3.99
III j	4OH, 3OCH_3	$C_{12}H_{11}N_3O_2S$	277.274	67	70	51.96	51.98	15.14	15.15	3.98	3.99
III k	4-OH	$C_{13}H_{13}N_3O_4S$	275.337	64	86	56.65	56.66	15.24	15.26	4.73	4.75
III l	-4-$N(CH_3)_2$	$C_{14}H_{16}N_4O_2S$	304.37	68	64	55.23	55.25	18.39	18.41	5.28	5.30
IV a	3-Cl	$C_{12}H_{12}lN_3O_2S$	297.76	71	70	48.38	48.40	11.90	11.91	4.04	4.06
IV b	2-Cl	$C_{12}H_{12}ClN_3O_2S$	297.76	72	75	48.39	48.40	11.90	11.91	4.05	4.06
IV c	4-Cl	$C_{12}H_{12}ClN_3O_2S$	297.76	73	78	48.38	48.40	11.89	11.91	4.05	4.06
IV d	2-No_2	$C_{12}H_{12}N_4O_4S$	308.31	70	90	46.73	46.75	18.68	18.70	3.91	3.92
IV e	3-No_2	$C_{12}H_{12}N_4O_4S$	308.31	72	92	46.74	46.75	18.69	18.70	3.90	3.92
IV f	4-No_2	$C_{12}H_{12}N_4O_4S$	308.31	69	88	46.75	46.75	18.70	18.70	3.92	3.92
IV g	-3Br	$C_{12}H_{12}BrN_3O_2S$	342.21	78	75	42.10	42.12	12.26	12.28	3.51	3.53
IV h	-4OCH_3	$C_{13}H_{15}N_3O_3S$	293.34	72	93	53.21	53.23	14.31	14.32	5.13	5.15
IV i	-2OH	$C_{12}H_{13}N_3O_3S$	279.31	67	70	51.58	51.60	15.03	15.04	4.67	4.69
IV j	4OH, 3OCH_3	$C_{12}H_{13}N_3O_3S$	279.31	69	72	51.59	61.60	15.03	15.04	4.67	4.69
IV k	4-OH	$C_{13}H_{15}N_3O_4S$	309.34	70	78	54.45	50.47	13.55	13.58	4.88	4.89
IV l	-4-$N(CH_3)_2$	$C_{14}H_{18}N_4O_4S$	306.28	65	78	54.86	54.88	18.25	18.28	5.92	5.92

Table 2
Antibacterial activity of the synthesized heterocyclic derivatives III (a-l) and IV (a-l) against various bacteria at two different concentrations (in ppm).

Comp.	*B . subtilis*		*E. coli*		*K. pneumoniae*		*S. aureus*	
	50	**100**	**50**	**100**	**50**	**100**	**50**	**100**
IIIa	++++	++++	+++	++++	++	++	++	+++
IIIb	++	+	++	++	+	++	++	+++
IIIc	+++	+++	+	++	+++	++	+	++
IIId	+++	++++	+	++	++	++	+	++
IIIe	++	+	+	++	++	+	+	++
IIIf	+	++	-	+	++	+	++	++
IIIg	-	++	+	++	+++	+++	+	++
IIIh	++	++	++	+	++	++	++	+
IIIi	-	+	++	+	++	++	+	++
IIIj	+	+	++	+	++	++	+	++
IIIk	++	+	+++	+++	+++	+++	+	++
IIIl	+++	+	+++	+++	+++	++	-	+
IVa	+++	++++	+++	+++	++++	+++	++	+++
IVb	++	++	+	++	++	+	++	+++
IVc	++	++	+	++	+	++	-	-
IVd	+	++	++	+++	+	+	++	+
IVe	-	-	+	+++	+	+	++	+
IVf	+	+	+	++	++	++	++	+++
IVg	+	+	+	++	++	++	+	++
IVh	++	++	-	+	++	+++	++	++
IVi	++	++	+	++	+++	+++	+++	+++
IVj	++++	+++	+	+++	+++	++	+++	++++
IVk	++	+	+	++	+	++	+	+
IVl	++	++	+	++	++	+++	++	++
Std.	+++	++++	+++	++++	++++	+++	++++	++++

Std.: Streptomycin : inhibition diameter in mm (-) 4; (+) 5-11; (++) 11-15; (+++) 15-19; (++++) 19-24.

Table 3

Antifungal activity of the synthesized heterocyclic derivatives III (a-l) and 4 (a-l) against various fungi at two different concentrations (in ppm).

Comp.	*A. flavus*		*A. niger*		*F. oxisporum*		*T. viridae*	
	100	**500**	**100**	**500**	**100**	**500**	**100**	**500**
IIIa	+++	++++	+++	+++	++	+++	++	+++
IIIb	+++	++++	+	++	++	++	+++	++++
IIIc	+++	+++	+++	+++	+++	+++	+++	+++
IIId	+++	+++	++	++	++	++	+	++
IIIe	+++	+++	++	++	++	++	+	++
IIIf	+	++	+	++	+	++	-	++
IIIg	+	++	+	+++	+	++	++	+++
IIIh	+	++	+	++	+	++	+	++
IIIi	-	+	++	+++	+	++	+	++
IIIj	+	++	+	++	+	++	++	+++
IIIk	-	+	+	++	+	++	++	++
IIIl	-	-	-	+	+	++	+	++
IVa	+++	++++	++	+++	+++	+++	+++	+++
IVb	+	++	-	+	+	++	++	+++
IVc	+	++	+	++	++	++	+	++
IVd	-	+	+	++	+	++	++	+++
IVe	+	+	+	++	+	++	+	++
IVf	+	++	+	++	+	++	+	+++
IVg	+	++	+	++	+	++	+	+++
IVh	+	++	++	+++	++	++	++	++
IVi	++	++	+	++	++	+	+++	+++
IVj	+++	++++	++	+++	+++	+++	+++	++++
IVk	-	+	-	+	++	++	+	++
IVl	+	++	-	++	++	+	++	++
Std.	+++	++++	+++	++++	++++	++++	+++	++++

Std. :Griseofulvin : inhibition diameter in mm(-)4 (+) 5-11; (++) 11-15;(+++) 15-19; (++++) 19-24.

Table 4

Insecticidal activity of synthesized heterocyclic derivatives III (a-l) and IV (a-l) against *Periplaneta americana* (KD value in minutes).

Compound Code	Time (in minutes)		Compound Code	Time (in minutes)	
	1%	2%		1%	2%
IIIa	10	9	IV a	7	6
IIIb	11	9	IV b	6	5
IIIc	12	10	IV c	6	5
IIId	8	5	IV d	9	8
IIIe	7	5	IV e	10	9
IIIf	9	4	IV f	9	7
IIIg	9	8	IV g	11	10
IIIh	15	10	IV h	18	12
IIIi	12	10	IV i	7	5
IIIj	11	9	IV j	25	18
IIIk	25	15	IV k	30	17
IIIl	28	20	IV l	18	15
Std.	7	5	Std.	7	6

Std : Cypermethrin is used as standard drug.

Table 5

Anthelminitic activity of synthesized heterocyclic derivatives III (a-l) and IV (a-l) against *Pheretima posthuma*. (Paralytic and lethal in minutes)

Compound Code	Paralytic (Time in minutes)	Lethal (Time in minutes)	Compound Code	Paralytic (Time in minutes)	Lethal (Time in minutes)
IIIa	3	5	IV a	4	5
IIIb	5	8	IV b	5	7
IIIc	6	9	IV c	3	5
IIId	4	6	IV d	4	6
IIIe	5	7	IV e	5	7
IIIf	8	11	IV f	8	9
IIIg	5	7	IV g	6	8
IIIh	6	8	IV h	7	9
IIIi	7	0	IV i	9	11
IIIj	8	9	IV j	9	10
IIIk	5	7	IV k	2	5
IIIl	10	12	IV l	3	7
Std.	2	5	Std.	3	6

Std. :Albendazole is used as standard drug.

The *Periplaneta americana* has been taken for insecticidal activity study and 1% and 2% solutions of prepared compound (0.1 mol) were injected in the abdominal region of cockroach with the help of micro syringe. The time of death was noted as KD (Knock Down) value. Standard drug *Cypermethrin* has been used under the similar conditions.

At the time of death the Antennae became motionless the appendages shrunk and folded towards the ventral side and cockroach lay dorsally.

The Indian adult Earthworm is taken for the anthelmintic activity. All the synthesized heterocyclic derivatives dissolved in minimum amount of DMF and then volume is adjusted to 10 ml with saline water. All drugs and solutions of synthesized heterocyclic derivatives were freshly prepared before starting the experiment groups of six earthworms were released into 10 ml of desired formulations.

Albendazole has been used as standard drug and used under similar conditions. Observations were made for the time taken to paralysis and death of individual worms. Paralysis was said to occur when the worm did not revive even in normal saline. Death was concluded when the worms lost their motility followed with fading away of their body colour[19,20], on going through the result of biological activity of 3-(substituted phenyl benzylidene amino)-1-acetyl-2-thioxo imidazolidine-4-one III(a-l) and N-(4-acetyl-4,5-dihydro-5-(substituted phenyl) 1,3,4-thiadiazole-2-yl) acetamide IV (a-l) showed that IIIa (3-Cl), IIIC (4-Cl) IIId (2-NO_2) IVa (2-Cl), IVj (4-OCH_3) are highly active against the selected bacteria and fungi and rest of the heterocyclic derivatives have shown good to moderate activity. All nitro derivatives of IIIrd series (IIId, IIIe, IIIf) and chloro (IVa, IVb, IVc) and hydroxyl group IV (i) (2-OH) have shown better insecticidal activity in comparison to the standard drug and rest of the synthesized heterocyclic derivatives have shown good to moderate activity.

IIIa (3-Cl) IIId (2-NO_2) and IVk (4-OH, 3-OCH3) IVl 4N(CH_3)$_2$ showed better anthelmintic activity in comparison to the standard drug and rest of the synthesized heterocyclic derivatives have shown good Anthelmintic activity. Thus we conclude that the synthesized heterocyclic derivatives may act as good antimicrobial agents, insecticidal and anthelmintics.

In this way the synthesized heterocyclics may prove fruitful to render the services to the humanity.

Acknowledgement

The authors are thankful to SAIF, Punjab University Chandigarh for providing the NMR spectrum and Analytical data of the synthesized heterocyclic derivatives, we are also thankful to Prof. U.S. Gupta, Deptt. of Zoology, Dr. H.S. Gour University Sagar (M.P.) for their kind assistance for performing Insecticidal and Anthelmintic activity in Zoology Department, Prof. M. Bhide, Department of Biotechnology, Dr. H.S. Gour University, for her kind help to conduct Antimicrobial activity and Prof. K.S. Pitre Head Department of Chemistry Dr. H.S. Gour University Sagar for providing IR spectral data and laboratory facilities.

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