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The views and opinions expressed in this journal are those of the contributors; Science-Tech Foundation does not necessarily concur with the same. All correspondence should be addressed to the Editor-In-Chief (Hon.), Journal of Pharmacy and Chemistry (Science-Tech Foundation), Plot No 22, Vidyut Nagar, Anantapur - 515 001, Andhra Pradesh, India. Phone: +91-8554 274677, Mobile: +91-94414 89324 • e-mail: jpcanantapur@gmail.com.

Microbial production of Poly (3-hydroxyalkanoate) s (PHAs) from various carbohydrates by *Pseudomonas* species ROU9 isolated from industrial polluted soils

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ABSTRACT

The increasing effect of non-degradable plastic wastes is a growing concern. Polyhydroxyalkanoates (PHAs), macromoleculepolyesters naturally produced by many species of microorganisms, are being considered as a replacement for conventional plastics. In this study, accumulated poly-(3-hydroxyalkanoates) (PHAs) was investigated in 14 strains of *Pseudomonas* species. The yield of PHAs produced by the strains according to dry cell mass ranged between 16-32%. *Pseudomonas* sp. ROU9, which produced the highest percentage yield of PHAs was examined for PHAs production over different incubation times (between 12 h and 84 h). Also, the amount of PHA produced by this isolated in different carbon and nitrogen sources was analyzed. In this strain, sucrose was the most efficient carbon sources for the production of PHA compared with other carbon (glucose, lactose, sucrose, pyruvate) and nitrogen (protease-peptone, L-glycine, L-cysteine, and $(NH_4)_2SO_4$) sources. The highest yield of PHAs production (32% on cell dry weight) was found at 1%w/v sucrose. This observation emphasizes the potential of the use of *Pseudomonas* in the commercial production of PHB and other PHAs.

Key words: PHAs, *Pseudomonas*, Liofilization, M17 broth medium, H[1] NMR

Introduction

Growth in the human population has led to the accumulation of huge amounts of non-degradable waste materials across our planet [1]. The accumulation of plastic wastes has become a major concern in terms of the environment [2, 3]. Conventional plastics not only take many decades to be decomposed in nature, but also produce toxins during the process of degradation. For this reason, there is special interest in producing plastics from materials that can be readily eliminated from our biosphere in an "environmentally friendly" fashion [4]. The allure of bioplastic is also linked to diminishing petrochemical reserves. The industrialized world is currently highly dependent on fossil fuels as a source of energy for industrial processes and for the production of structural materials [1]. Fossil fuels are, however, a finite resource and current evidence suggests, based on recent usage trends and the rate of discovery, that utilization rates will outstrip discovery from about 2010 [5]. This is a global problem as our economy is still very oil dependent. The world currently consumes approximately 140 million tons of plastics per annum [1]. Processing of these plastics uses approximately 150 million tons of fossil fuels, which are difficult to substitute. The challenge to the world is whether we can substitute the source of these long carbon arrays from a non-sustainable source with a sustainable renewable one [1].

Due to its biodegradable nature, PHA has a broad range of applications, including medical applications such as wound management, drug delivery, and tissue engineering [6, 7]. Furthermore, PHA is composed of chiral hydroxy acids that have potential as synthons for anti-human immunodeficiency virus drugs, anticancer drugs, antibiotics, and vitamins [8, 9].

Polyhydroxyalkanoates (PHAs) are biopolymers which are synthesized by some bacteria and accumulated intracellularly. They are widely studied as a biodegradable and biocompatible plastic polymer. *Pseudomonas* strains known to accumulate PHA, such as *P. fluorescens* PfO-1, *P. aureofaciens* and *Pseudomonas* sp.61-3. During growth of *Pseudomonas oleovorans* on nitrogen limited medium poly

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(3-hydroxyalkanoate) (PHA) is accumulated as intracellular storage material. PHA is a high molecular weight polymer and a fully biodegradable plastic, with interesting application possibilities. The intracellular formation of PHA is catalyzed by PHA polymerase, which esterifies 3-hydroxyacyl-coenzymeA into PHA polymer [10]. This study describes the screening for PHA-producing *Pseudomonas* sp.. isolated from industrial polluted soils Bacterial isolates obtained from various locations around Hyderabad industrial area were tested for PHA production. We describe a strain of *Pseudomonas* sp. ROU9 capable of accumulating PHA and which may allow the development of novel culture methods for biodegradable microbial polyesters production from residue.

Material and Methods

Microorganisms (Bacteria)

The species of *Pseudomonas* used in this study were isolated from various locations of industrial polluted soils at industrial development area, Hyderabad. To select the potential PHA accumulating isolate, the bacteria are grown in E2 medium. All of the strains were stored at –80°C in nutrient agar medium with 10% v/v glycerol, and regenerated twice before use in the manipulations.

Media and growth conditions

Pseudomonas sp. ROU9 cultures were grown in rotary 250-ml conical flasks containing 50 ml of E2 medium (Vogel and Bonner. 1956) at 30°C, with shaking at 230 rpm. *Pseudomonas* sp. ROU9 cells were first grown in E medium [11] supplemented with 0.1% microelement solution [12] to produce the biomass. The cells were harvested after 20 h by centrifugation, washed and then transferred to the nitrogen-limiting E2 medium [12] containing a specified carbon substrate at a concentration of 1 to 3% (w/v). Cells were harvested after 48 h by centrifugation, washed and lyophilized.

PHA recovery

Cells were collected by centrifugation and liofilization. This biomass was determinated by gravimetry. 0.2g of biomass was suspended in 5 mL of 0.2% (w/v) sodium hypochlorite. After 1 h at 37ºC, to allow the total lysis of the suspension, PHA granules were collected by centrifugation (2000 x g). The pellet was washed with distilled water, acetone and ethanol, and the final pellet was dissolved in chloroform. The chloroform was evaporated at room temperature and PHA weight was obtained by gravimetry [13]. Polymer identification and chemical characterization was analyzed with IR and NMR spectroscopy.

Effect of Production of PHB in Different Carbon and Nitrogen Sources

PHB productions of strain showing the highest PHB production were determined in different carbon and nitrogen sources. Yeast extract was taken out, and carbon sources (glucose, lactose, sucrose and pyruvate, 1% w/v) were added into M17 broth medium. Peptone in M17 broth medium was taken out, and nitrogen sources (Protease peptone, L-glycine, L-cysteine, and (NH4)2SO4, 1% w/v) were added. Nitrogen and carbon sources were sterilized by Millipore filter with a pore size of 0.45 µm.

Results

Microorganisms

Selection and Identification of the potential PHA accumulating bacterial isolates:

After quantitative estimation of the amount of PHA accumulated by these 14 isolates (Table. 1) using Law and Slepecky method, only ROU9 isolate that was accumulating PHA in sufficiently large amounts was selected. The well responded isolate was identified as *Pseudomonas* sp. after performing biochemical characterization and named the strain as ROU9 and accumulated PHAs by ROU9 was characterized as per the requirements.

Chemical characterization of PHA

IR spectra

IR spectra were recorded for PHA dissolved in chloroform. Spectra of the standards PHB and P(HB-co-HV(12%)) both showed two intense absorption bands at 1724.3 cm^{-1} and 1280.3 cm^{-1} corresponding to C=O and C-

Table-1

Accumulation of PHAs and yield percentage based on cell dry weight by the selected (fourteen) isolates of *Pseudomonas* bacteria

Nmber of Isolates	Strain Number	PHAs Yield in % (CDW)
1	ROU1	22.1
2	ROU2	16.0
3	ROU3	29.6
4	ROU4	18.0
5	ROU5	20.7
6	ROU6	18.1
7	ROU7	30
8	ROU8	19.5
9	ROU9	32.0
10	ROU10	23.0
11	ROU11	28.9
12	ROU12	23.7
13	ROU13	29.0
14	ROU14	27.8

CDW: Cell Dry Weight

O stretching groups respectively. The polymer from isolate ROU9 *(Pseudomonas* sp..*)* when grown on glucose as sole carbon source exhibited a sharp peak at 1714 cm^{-1}, corresponding to the C=O stretching. The peak at 1190 cm^{-1} confirmed C-O stretching in the polymer as ester of HA. The IR spectra of the polymer are shown in figures 1.

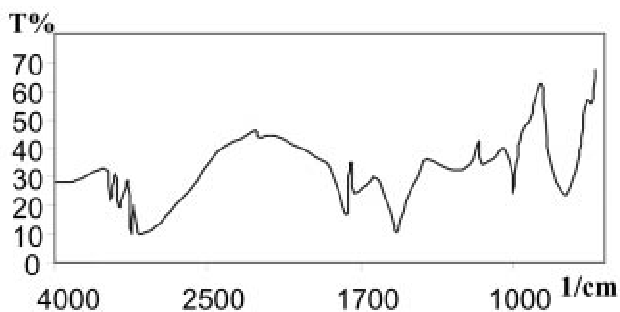

Fig.1: IR spectra of polymer from *Pseudomonas* sp. ROU9 grown on Glucose

H^1 NMR

The H^1 NMR scans of the polymers from isolates ROU9 *(Pseudomonas* sp.*)* and was recorded. The H^1 NMR spectra were used to determine the monomer composition of the polymers produced by these isolate. The figure 2 shows the H^1 NMR spectra of the polymer from the isolate, when grown on glucose as carbon source.

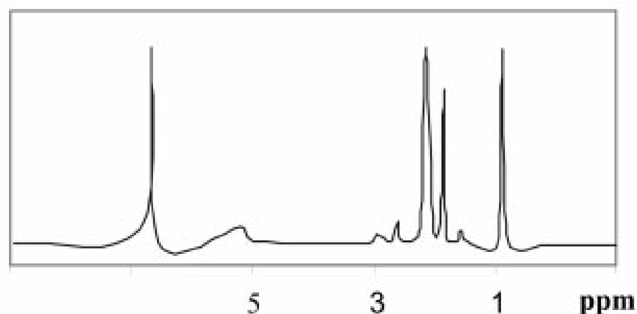

Fig.2: NMR of polymer from *Pseudomonas* sp. ROU9 grown on glucose.

Effect of Production of PHB in different Carbon and Nitrogen Sources

PHB production of *Pseudomonas* ROU9 showing the highest PHB production was determined in different carbon and nitrogen sources. The results are indicated in figure 3 and 4. The strain produced PHB in M17 containing each of the sugars tested; pyruvate was the poorest carbon sources (10.1%), and sucrose was by far the most efficient carbon source (31.8%). The amount of PHB produced by this strain in different nitrogen sources was lower than the amount of PHB production in M17 broth medium (control) (figure 4).

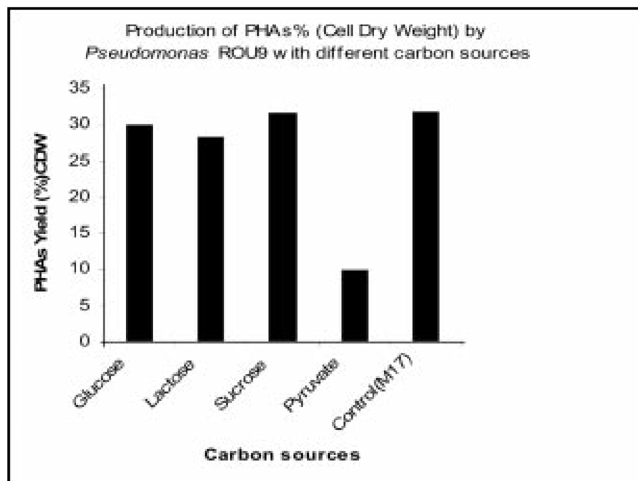

Fig.3: Production of PHAs % (CDW) by *Pseudomonas* ROU9 with different carbon sources

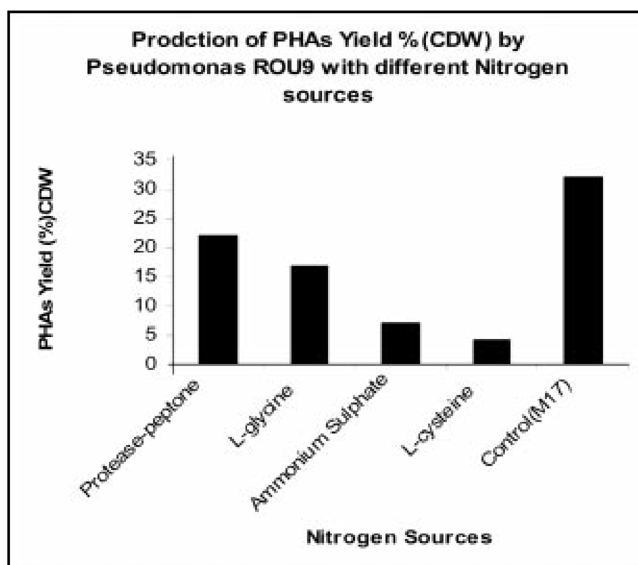

Fig.4: Production of PHAs % (CDW) by *Pseudomonas* ROU9 with different Nitrogen sources

Discussion

Bacterial polyhydroxyalkanoates (PHA) have attracted much attention as environmentally degradable thermoplastics. They are being viewed as potentially useful for replacing many synthetic plastics in a wide range of agriculture, marine and medical applications. While the conventional synthetic plastics contribute to serious pollution problems, PHA could be broken down by some microorganisms, such as bacteria and fungi, which are able to secrete extracellular PHA depolymerases.

Unlike petroleum-derived plastics that take several decades to degrade, PHAs can be completely bio-degraded within a year by a variety of microorganisms [1]. This biodegradation results in carbon dioxide and water, which return to the environment. Attempts based on various methods have been undertaken for mass production of PHAs. Promising strategies involve genetic engineering of microorganisms and plants to introduce production pathways [1].

In this study, biodegradable polyhydroxyalkanoate polyesters were produced using *Pseudomonas* sp. which were extracted, and after purification, they were characterized. Polymer Production-In these preliminary studies, our aim was to determine the optimum growth and production conditions. A total of 14 strains of *Pseudomonas* sp. were determined for the production of PHAs in M17 medium. PHAs produced by the strains is exhibited in Table 1. The highest PHAs production and productivity percentage were found in *Pseudomonas* sp. ROU9 (32% w/w CDW). The lowest PHB production was obtained in *Pseudomonas* sp.ROU2. (16.0%) Also, it was investigated whether any relationship between the dry cell weight and PHA production existed. PHAs production of *Pseudomonas* sp. showing the highest PHA production was determined in different carbon and nitrogen sources. The results are indicated shown in figure 3 and 4. The strain produced PHA in M17 containing each of the sugars tested: pyruvate was the poorest carbon sources where as sucrose was by far the most efficient carbon source.

The thermoplastic properties of the polymer and its biodegradability determine its importance as a substitute for petrochemical plastics [14]. Different amounts of PHB were produced by the strains studies; however, PHAs levels were 16 to 32% in M17 medium [15] mentioned that the amount of PHB produced by some *Lactobacillus* species was higher than that produced by *Lactococcus, Pediococcus* and *Streptococcus strains.* Some of the *A. eutrophus* strains used for commercial PHB production have a PHB concentration which is approximately 80% (w/w) of the dry cell weight [6, 16] studied PHA in 11 different Bacillus sp.. and found PHB consisting 50% (w/v) of dry cell weight of the bacteria.

In one of the studies conducted by Aslim and co workers [15] it was reported the production of PHB by some lactic acid and *Pseudomonas* bacteria and found the highest value of PHB was 35.80% (w/v) (for Lb. bulgaricus C8) for dry cell weight. When compared to related literature, our results show a lower PHB production. The differences above, we think, were resulted from different strains, types of medium and cultivation method used in individual study. In recent years, a combination of genetic engineering and molecular microbiology techniques has been applied to enhance PHA production in microorganisms. Several mutants with phenotypes in PHA synthesis were characterized in order to develop optimal recombinant host strains [1]. Over-expression of pha genes in the natural PHA producer, however, resulted in little difference in polymer accumulation [1].

References

[1] Pornpa S, Randall W, Suresh N, Maurice M, and Saleh S. Biotechnol Adv 2007 :25;148–175

[2] Guillet J. Plastics and environment. In: Scott G, editor. Degradable polymers: principles and applications. Dordrecht, The Netherlands: Kluwer Academic Publisher; 2002. p. 413–48.

[3] Thompson RC, Olsen Y, Mitchell RP, Davis A, Rowland SJ, John AWG, et al. Science 2004;304:838.

[4] Gross RA, Kalra B. Science 2002;297:803–7.

[5] Zagar J. The end of cheap conventional oil. The Proceedings of Energy Efficiency Policy Synoposium; 2000. www.hawaii.gov/dbedt/ent/ symposium/zagar.pdf.

[6] Lee SY. Bacterial polyhydroxyalkanoates. Biotechnol Bioeng 1996;49:1– 14.

[7] Zinn M, B Witholt, T Egli. Adv Drug Delivery Rev 2001;53: 5–21.

[8] De Roo G, MB Kellerhals, Q Ren B, Witholt B Kessler. Biotechnol Bioeng 2002;77:717–722.

[9] Ohashi T, and J Hasegawa. 1992. New preparative methods for optically active hydroxycarboxylic acids, p. 249–268. In G. N. Sheldrake, A. N. Collins, and J. Crosby (ed.), Chirality in industry. John Wiley & Sons, New York, N.Y.

[10] Kraak, Marjan Nienke. 1998. http://e-collection.ethbib.ethz.ch/view/eth:41186) accessed on 12/5/2009.

[11] Vogel HJ, DM Bonner. J Biol Chem 1956;218:97–106.

[12] Lageveen RG, Huisman GW, Preusting H, Ketelaar P, Eggink G, Witholt B Appl Environ Microbiol 1988;54: 2924-2932

[13] García Lillo J, Rodríguez Valera F. App Env Microbiol 1990;56:2517-2521.

[14] Celik GY, Beyatli Y, Aslim B. Fresenius Env Bull 2005;14:954-956.

[15] Aslim B, Çalişkan F, Beyatli Y, Gündüz U. FEMS Microbiol Let 1998;159: 293-297.

Studies on antimicrobial screening of Thiosemicarbazones and their metal complexes

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ABSTRACT

A few thiosemicarbazone ligands and their Fe(III), Co(II), Ni(II), Cu(II), Zn(II), Pd(II), Cd(II) and Hg(II) complexes have been screened for antimicrobial activity against some gram (+ve) and gram (-ve) bacterial and fungal species. The metal complexes are found to have higher activity than the ligands. Further, the metal complexes exert differential activity against the organisms studied and the results obtained are discussed.

Keywords: Metal – Thiosemicarbazone complexes, antimicrobial activity

Introduction

Thiosemicarbazones constitute a special class of organic compounds owing to their chemical and biological importance. These compounds which are potential chelating agents endowed with diverse ligating behaviour [1, 2] have been reported to possess a wide spectrum of medicinal properties [3-6]. The biological activity of these compounds has been attributed to their ability to chelate trace metal ions and, in many a case, the metal ion association exerts a synergistic effect on the activity of the free ligands [7,8]. In this paper, we report the results on the antimicrobial screening of thiosemicarbazone ligands namely 2 - aminonicotinaldehyde thiosemicarbazone (ANTSC), 3-formylchromone thiosemicarbazone (FCTSC), 2-hydroxy–3–methoxybenzaldehyde thiosemicarbazone (HMBTSC) and 2,4- dihydroxyacetophenone thiosemicarbazone (DPTSC) and their Fe(III) , Co(II), Ni(II), Cu(II), Zn(II), Pd(II), Cd(II) and Hg(II) complexes against gram (+ve) bacterium:Bacillus subtilis, gram (-ve) bacterium : *Escherichia coli* and fungus: *Aspergillus niger*.

Experimental

Preparation of ligands and their metal complexes

The synthesis and characterization of the ligands and their metal complexes employed in the present study have been reported earlier [2].

Preparation of test samples

The test solutions of the samples were prepared in dimethylformamide. The antibiotics: benzyl penicillin and streptomycin sulphate were used as standards for antibacterial screening and nistatin was used as a standard for antifungal screening.

All the samples under present investigation were dissolved in DMF to give a concentration of 1 mg / ml. The antibacterial standards were dissolved in sterile distilled water. The antifungal standard was dissolved in buffered 70 % propanol.

Test microorganisms

The micro organisms employed in the study were *Bacillus subtilis* (gram +ve) and Escherichia coli (gram –ve) bacteria and fungus: *Aspergillus niger* .

Preparation of inoculum and nutrient medium [9, 10]

Nutrient broth (P^H – 7.2) was used for the preparation of inoculum of bacteria. The composition of broth was peptone 5.0. g, Beef extract 1.5 g, Yeast extract 1.5 g and distilled water 1000 ml Nutrient broth was used for the preparation of medium for antibacterial screening. The medium contained 1.5% of agar in addition to the composition of nutrient broth.

For antifungal screening, inoculum was prepared by transferring a loopful of stock culture (Glucose – 40 g, Peptone – 10 g, Agar – 20 g, distilled water up to 1000 ml) to a 125 ml Erlenmayer flask containing 80 ml of Sabouraud's broth. The composition of broth is same as that of stock culture with the exception of agar .The inoculum flask was incubated for 18 hours at 25° C and stored at 50°C.

Preparation of plates

For antibacterial screening, the agar medium was sterilized by autoclaving at 121° C for 15 minutes. The petri

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plates and pipettes plugged with cotton were sterilized in an oven at 150° C for one hour. About 25 ml of the molten medium was poured in each of sterilized petri plates. About 0.5 ml of 24 hours old broth cultures of bacterial strains were added to the respective petri plates. The contents of Petri plates were mixed thoroughly by rotary motion. After solidification of the medium, four cups (diameter 8 mm) were made with the help of a sterile borer at equal distances.

For antifungal activity, the corning sterile Petri plates were used for investigation. About 20 ml of previously inoculated Sabouraoud's agar medium was poured in it. After solidification of the medium, four cups (diameter 8 mm) were made with the help of a sterile borer at equal distances.

Accurately measured 0.1 ml of samples and 0.1 ml of standard antibiotics were added into the cups and labelled accordingly. The plates were kept undisturbed in a cool place for one hour to allow the solutions to diffuse into the medium. The plates were then incubated at 37° C for 24 hours.

For antifungal screening, each cup in petri plate was loaded with 0.1 ml of respective solutions. The plates were kept undisturbed in a cool place for 2 hours to allow the solutions to diffuse into the medium and then incubated at 25° C for 24 hours.

Measurement of activity

The presence of a definite zone of inhibition surrounding the cups indicated antimicrobial activity. The diameter of the zone of inhibition was recorded. The experiments were performed, at least, in triplicate.

Dose dependent activity of compounds

Based on antimicrobial profiles, further studies were carried out to find out dose dependent activity of the selected compounds. Four different concentrations of test samples (0.5,1.0, 2.0 and 3.0 mg/ml) and standard antibiotics (50, 100, 200, and 500 μg/ml) were employed in assessing the level of antimicrobial activity of the compounds. Accurately measured 0.1 ml of the test and standard solutions were placed in cups prepared in seeded agar petri plates as described earlier. The petri plates were left undisturbed in a cool place for one hour to allow proper diffusion and then incubated at 37° C for 24 hours in the case of bacteria and at 25° C for 48 hours in case of fungus. After the incubation period, the diameter of zone of inhibition was measured with antibiotic – zone reader and the experiments were carried out in triplicate. The activities of the compounds are compared with those of the respective standards.

Results

The antimicrobial screening of the ligands and their metal complexes has been first carried out on Bacillus substilis(Gram +ve) and Escherichia coli(Gram –ve) bacteria and Aspergillus niger (fungus) to find out the activity spectrum of the compounds. A zone of inhibition of 20 mm or above has been considered as a significant activity. The results are presented in Table 1.

It is observed that none of the ligands is associated with considerable antimicrobial activity, while the metal complexes screened possess higher activity than the ligands. The Co(II), Ni(II) and Hg(II) complexes of ANTSC, the Ni(II), Cu(II) and Pd(II) complexes of HMBTSC and the Ni(II) and Cu(II) complexes of DPTSC have been associated with significant activity.The results in the table indicate that the activities of the complexes against the bacteria and fungus studied are comparable with a small variation either way.

The set of eight complexes that is significantly active against *Bacillus subtilis*, *Escherichia coli* and *Aspergillus niger* at the concentration 1mg/ml (Table-1) has been subjected to dose-dependent study against the same microorganisms employing four different concentrations i.e., 0.5, 1.0, 2.0 and 3.0 mg/ml in DMF. The concentrations of the standards that have been used correspond to 50, 100, 200 and 500 μg/ml and the results obtained are presented in the Tables 2a -- 2c.

Discussion

The results in the Tables 2a--2c indicate that the compounds are associated with the least activity at the lowest concentration. The activity of the compounds, in general, increases with increase in their concentration even though the increase is not proportional. Most of the compounds screened show jump in activity when the concentration is increased from 0.5mg/ml to 1mg/ml. And, thereafter, as the concentration is raised, the activity remains the same or the increase, if there is any, is only marginal. These observations recommend the optimum concentration of the compounds for activity to be 1mg/ml.

From the results, it is known that the metal complexes exert more of antimicrobial activity than the ligands indicating that the metals are actually in action [11,12]. It is assumed that the role of ligands associated with the metal ion in a complex is to make the metal ion fat soluble. Once the complex makes its way across the cell membrane, it, depending on the environment, undergoes dissociation releasing the metal ion and enabling it to exert its toxic effect. The differential activity associated with the complexes may be traced to this.

Table : 1

Antimicrobial activity of the ligands and their metal complexes concentration : 1 mg/ml in DMF; Zone of inhibition in mm.

Sl. No.	Complex	Zone of Inhibition		
		Bacillus subtilis	*Escherichia coli*	*Aspergillus niger*
1	Fe-ANTSC	12 (10)	11 (10)	11 (10)
2	Co-ANTSC	26	20	21
3	Ni-ANTSC	21	22	23
4	Cu-ANTSC	12	11	13
5	Zn-ANTSC	11	11	12
6	Pd-ANTSC	11	12	12
7	Cd-ANTSC	11	11	11
8	Hg-ANTSC	21	20	21
9	Fe - FCTSC	14 (10)	15 (12)	12 (10)
10	Co-FCTSC	12	12	14
11	Ni-FCTSC	11	13	12
12	Cu-FCTSC	11	13	12
13	Zn-FCTSC	12	14	14
14	Pd-FCTSC	12	13	11
15	Cd -FCTSC	11	13	12
16	Hg-FCTSC	12	14	11
17	Fe – HMBTSC	12 (10)	13 (12)	11 (10)
18	Co –HMBTSC	11	13	12
19	Ni-HMBTSC	28	24	25
20	Cu-HMBTSC	27	28	29
21	Zn-HMBTSC	14	13	11
22	Pd-HMBTSC	20	21	23
23	Cd-HMBTSC	12	14	13
24	Hg-HMBTSC	11	13	12
25	Fe – DPTSC	13 (12)	11 (10)	12 (11)
26	Co-DTPSC	14	12	13
27	Ni-DTPSC	24	22	21
28	Cu-DTPSC	20	23	22
29	Zn-DTPSC	13	12	12
30	Pd-DTPSC	14	11	12
31	Cd-DTPSC	13	12	12
32	Hg-DTPSC	14	11	12

The values in parentheses correspond to those of the ligands.

Table : 2a

Antimicrobial activity of the metal complexes
Microorganism : *Bacillus subtilis* ;
Standard : Benzyl penicillin

Sl. No.	Compound	Zone of inhibition in mm.			
		A	B	C	D
1	Co-ANTSC	27	26	26	16
2	Ni-ANTSC	26	23	21	14
3	Hg-ANTSC	24	22	20	18
4	Ni-HMBTSC	30	29	28	16
5	Cu-HMBTSC	28	28	27	20
6	Pd-HMBTSC	23	22	20	15
7	Ni-DPTSC	31	25	24	21
8	Cu-DPTSC	28	27	20	17
9	Standard	24	21	20	16

Table: 2c

Antimicrobial activity of the metal complexes
Microorganism: *Aspergillus niger*;
Standard: Nistatin

Sl. No.	Compound	Zone of inhibition in mm.			
		A	B	C	D
1	Co-ANTSC	22	21	20	14
2	Ni-ANTSC	22	21	20	12
3	Hg-ANTSC	21	20	20	18
4	Ni-HMBTSC	28	27	25	19
5	Cu-HMBTSC	35	33	29	22
6	Pd-HMBTSC	24	23	23	18
7	Ni-DPTSC	22	22	21	19
8	Cu-DPTSC	27	25	22	19
9	Standard	25	20	18	17

* Test Solution	* Standard Soluation
A = 3.0 mg/ml	A = 500 µg/ml
B = 2.0 mg/ml	B = 200 µg/ml
C = 1.0 mg/ml	C = 100 µg/ml
D = 0.5 mg/ml	D = 50 µg/ml

(* for Tables - 2a, 2b and 2c)

Table: 2b

Antimicrobial activity of the metal complexes
Microorganism : *Escherichia coli*;
Standard : Streptomycin sulphate

Sl. No.	Compound	Zone of inhibition in mm.			
		A	B	C	D
1	Co-ANTSC	29	28	20	17
2	Ni-ANTSC	24	24	22	15
3	Hg-ANTSC	24	21	20	13
4	Ni-HMBTSC	26	25	22	15
5	Cu-HMBTSC	34	32	28	21
6	Pd-HMBTSC	23	22	20	15
7	Ni-DPTSC	25	24	22	16
8	Cu-DPTSC	24	24	23	20
9	Standard	26	24	20	16

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References:

[1] Padhye. S and Kouffman GB. Elsevier Science publishers. B.V 1985;127

[2] Ramachary M, Sudershan T, Laxma Reddy K, Sri Hari S. Asian J Chem 1989; 1: 239.

[3] Huang ES Virol J 1973; 61: 1560.

[4] Nyomol.O, Thorlog-Lewson DA, Elkington. J, Strominger JL. Nat Acad Sci 1961; 73: 1744.

[5] Klayman DL, Bartosevich JE, Griffin TS, Mason CJ, Covill JP. J Med Chem 1979;22:855.

[6] Petering HG, Buskirk HH, Underwood GE, Cancer Res1964;64:367.

[7] Liebermeister K. Naturforish Z 1950;85:79.

[8] Mikelens PE, Woodson BA, Levinson WE. Biochem Pharmacol 1976; 25; 821.

[9] Kavanagh F in "Analytical Microbiology"1963; 403.

[10] Burdon KL in "Introduction to Microbiology" The Macmillan Co., NewYork, 1968; 102.

[11] Gilman AG, Goodman LS and Gilman A, The Pharmacological basis of therapeutics, Macmillan publishing co.,Inc.,6th ed., 1980;1623.

[12] Perrin DD and Agarwal RP in Williams(eds).D.R "An Introduction to Inorganic Chemistry, Thomas, Springfield,Illinois, 1976;361.

An overview of *Chenopodium album* (Bathua sag) chemistry and pharmacological profile

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ABSTRACT

Chenopodium album from family Chenopodiaceae known as Bathua sag is being used in traditional medicines. It has been found to have anthelmentic, laxative, blood purifier, stomach worms, diuretics, abdominal pain and aphrodisiac activity. It has been also found to contain essential oil, alkaloid, quercetin, choline, vitamin C, potassium, fats, carbohydrates, minerals, and ascaridole. An attempt has been made to provide up to date references that are categorized on the basis of phytochemical and pharmacological activities.

Introduction

A genus of strong smelling herb commonly known as pigweed in English and distributed through out world. About 21 species occur in India of which few have been introduced. Some are cultivated for vegetable, and few for grains. In Hindi common name is Bathua sag. A polymorphous, mealy white, erect herb, up to 1.5 ft in height, and found wield in altitude of 700 meters and cultivated through out India. Stems rarely slender, angled, often striped green, red or purple; leaves rhomboid, deltoid to lanceolate, upper entire, lower toothed or irregularly lobed, extremely variable in cultivated forms, 10-15 cm long, petioles often as long as thick blade; flowers in clusters forming a compact or loosely panicle spikes in axils; utricles with round, compressed, shining black seeds, possessing sharp margins.

The herb is a common weed during summer and winter in waste places and in the field of wheat, barley, mustard, gram and reduces their yield. The weeds are low growing while the cultivated plants are tall and leafy. The young plant of not more than 20 cm is much esteemed as a potherb. The tender shoots are eaten raw in salad or with curd; they are also cooked as vegetable or the cooked shoots are mixed with curd and eaten. The dried herb is stored for future use. It is also used as fodder; pigeons consume the plant in large quantities. The leaves are rich in potassium and vitamin C.

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Chemically leaves found to contain various components such as moisture (89.65), protein (3.7%), fat (0.4%), other carbohydrates (2.9%), minerals (2.6 gm), calcium (15gm), phosphorus (8 gm), iron (4.2 gm), thiamine (0.01mg), niacin (0.6 mg), vitamin C (35 mg), carotene (1.470 µg), along with traces of iodine, fluorine, and vitamin K. The essential amino acids of leaf proteins were as follows leucine, isoleucine, lysine, methionine, phenylalanine, threonine, valine and tryptophan [1].

Pharmacological aspect:

Antipruritic and antinociceptive activities

The ethanolic extract from the fruits of *Chenopodium album* L. (FCAL), orally administered at doses of 100-400 mg/kg, dose-dependently inhibited scratching behavior induced by 5-HT (10 micro g per mouse, s.c.) or compound 48/80 (50 micro g per mouse, s.c.) in mice. But it failed to affect hind paw swelling induced by 5-HT or compound 48/80 in mice at doses of 100 and 200 mg/kg and only showed a relatively weak inhibition on the swelling at a higher dose of 400 mg/kg. In addition, FCAL (200 and 400 mg/kg) significantly attenuated the writhing responses induced by an intraperitonial injection of acetic acid and the inflammatory pain response induced by an intraperitonial injection of formalin in mice. At a dose of 400 mg/kg, it also inhibited the neurogenic pain response of formalin test. In conclusion, FCAL possesses antipruritic and antinociceptive activities and the antinociceptive effects are not secondary to anti-inflammatory effects. The findings support evidence for the clinical use of FCAL to treat cutaneous pruritis [2].

Spermicidal activity

Aqueous decoction of *Chenopodium album* seeds (CAD) was assessed for its sperm-immobilizing and contraceptive efficacy in laboratory mammals. Spermicidal efficacy was evaluated in vitro by a modified Sander-Cramer test. The mode of spermicidal action was assessed by (a) supravital and double fluoroprobe staining of sperm, (b) hypo osmotic swelling tests and (c) transmission electron microscopy. Contraceptive efficacy was evaluated by intrauterine and vaginal application of CAD in rats and rabbits, respectively, followed by their mating and evaluation of pregnancy outcomes. The minimum effective concentration of CAD that induced instantaneous immobilization of rat spermatozoa in vitro was 2 mg/ml. The mechanism of CAD action involved disintegration of sperm plasma membrane and dissolution of acrosomal cap causing sperm death. Fertilization of oocytes and establishment of implantation were prevented in the uterine horn that was administered with CAD, while these events occurred unhindered in the untreated contra lateral side. In rabbit, intravaginal application of CAD significantly blocked the establishment of pregnancy. CAD possesses appreciable spermicidal potential, which may be explored as an effective constituent of vaginal contraceptive [3].

Phytochemical aspects:

The chemical examination of seeds of broad leaf diploid cytotype of *C. album* showed the presence of cryptomeridiol and 8- alpha-acetoxycyryptomeridiol. The growth promoting activity of the two compounds was studied. [4] A new phenolic amide has been isolated from roots of N-trans-feruloyl-4-o-methyldopamine by spectroscopic evidence and chemical synthesis. It showed attracting activity towards the zoospores of (*Aphanomyces cohlioides*) a pathogenic fungus against some plants of Chenopodiaceae. [5] TLC investigation of the flavonoid fraction of *C murale* revealed the presence of three components, major one identified as kaempferol-3,7-dirhamnoside 1 (kampferitrin; lespedin). Kampferitrin as well as the total flavonoid mixture, showed dose related hypotension and bradycardia in rabbit. It also produced a dose related hypotension in genetically prone hypertensive rats. Quantification of kaempferitrin in (C. *ambrosoides, C. ficifolium*) and (*C. album*) reveled that the former contains that the highest level. [6] Isolation and spectral data of three saponins, 3-o-beta-D-glucuronopyranosyl oleanolic acid or calenduloside E, 3-o-(beta-d-glucuronopyranosyl-28-o-beta-D-glucuronopyranosyl)-28-o-beta-D-ucopyranosyl oleanolic acid or chikusetsusaponin IV a and 3-0-{3' -o-(2''-o-glycolyl)-glyoxylyl beta –D-glucuronopyranosyl} oleanolic acid isolated from the roots of *C. album* are reported [7]. Bathua and spinach leafs containing 20.63 and 26.54 mg iron/100gm on dry weight basis were used for estimation of in vitro and in vivo availability of iron. In vitro availabilities of iron were 2.79 and 3.03 5 of total iron in bathua and spinach leafs respectively. The haemoglobin regeneration efficiency ratio of fats fed of bathua; spinach and synthetic diet were 19.60, 21.77 and 38.45 % respectively. Relative efficiency of haemoglobin regeneration was 52.31 and 54.44 in bathua and spinach fade rats, respectively. The highest apparent iron absorption was observed in rats fade on synthetic diet and the lowest in rats fade of spinach diet [8]. Seven cinnamic acid amides have been isolated from *Chenopodium album*. The structures have been attributed by means of their spectral data. One of them, N-trans-4-O-methylferuloyl 4'-O-methyldopamine is described for the first time. Their effects on germination and growth of dicotyledons *Lactuca sativa* L. (lettuce) and *Lycopersicon esculentum* L. (tomato) and of monocotyledon *Allium cepa* L. (onion) as standard target species have been studied in the range concentration 10(-4)-10(-7) M [9]. The roots of *Chenopodium album* were infused in methanol, and the extract was partitioned between ethyl acetate and water. Ethyl acetate soluble material was subjected to different silica-gel column chromatography and then purified by reverse-phase HPLC to afford a new cinnamic acid amide alkaloid as a racemic mixture. The new compound, named chenoalbicin (1) was characterized by extensive spectroscopic investigation, especially 1D and 2D NMR spectroscopy. Its effects on the germination and growth of *Lactuca sativa* L. were studied. The results were reported as percentage differences of germination, root elongation, and shoot elongation from the control at concentrations ranging from 10(-4) to 10(-7) M [10]. Two new compounds (1, 2) and 16 apocarotenoids (3-18) were isolated from the weed *Chenopodium album*. The structures of new apocarotenoids were determined to be (3R, 6R, 7E, 9E, 11E)-3-hydroxy-13-apo-alpha-caroten-13-one (1) and (6S, 7E, 9E, 11E)-3-oxo-13-apo-alpha-caroten-13-one (2) by spectroscopic, NMR, and MS analysis. Five of the known compounds (5, 6, 13, 15, and 17) were previously reported only as synthetic compounds. Effects of these compounds on germination and growth of *Lactuca sativa* (lettuce) were studied in the 10(-4)-10(-7) M concentration range [11]. The isolation and the structure determination of an unusual xyloside from the plant *Chenopodium album* are reported. The structure has been attributed by means of its spectral data [12]. Cinnamic acid, 4-hydroxy-cinnamic acid, ferulic acid, methyl ferulate, sinapic acid, methyl 3-(4-hydroxy-3-methoxyphenyl) propanoate, 4-(1-hydroxyethyl)-2-methoxyphenol, vanillyl alcohol, 4-(hydroxymethyl)-2-methoxyphenol, 4-hydroxy-3-methoxybenzoic acid, 4-vinylphenol, 4-methylbenzaldehyde, N-[2-(1H-indol-3-yl)ethyl]acetamide, pinoresinol, syringaresinol, lariciresinol, 5,5'-dimethoxy-lariciresinol, threo-guaiacylglycerol-3-beta-4-syringaresinol ether and two new sesquilignans, namely, threo-guaiacylglycerol-alpha-O-methyl-beta-O-4-syringaresinol ether and threo-syringylglycerol-alpha-O-methyl-beta-O-4-syringaresinol ether, were isolated and identified as components of *Chenopodium album*. Constitutions were established based on spectroscopic data, including two-dimensional NMR analyses [13].

References

[1] Kirtikar KR and Basu BD. Indian Medicinal Plants: Lalit Mohan Basu, Allahabad, Vol III, 2 nd Edn: 1935; 1964-1965.

[2] Dai Y, Ye WC, Wang ZT, Matsuda H, Kubo M, But PPH. J Ethnopharmacol 2002;81(2): 245-250.

[3] Kumar S, Biswas S, Mandal D, Roy HN, Chakraborty S, Kabir SN, Banerjee S, Mondal NB. Contraception 2007; 75(1): 71-8.

[4] Bera B, Mukherjee KK, Ganguly SN. Fitoterapia 1991; 62(2):178.

[5] Horio T, Yoshida K, Kikuchi H, Kawabata J, Mizutani J. Phytochem, 1993, 33(4): 807-808.

[6] Gohar AA, Elmazar MMA. Phyt Res 1997:11(8): 564-567.

[7] Lavaud C, Voutquenne L, Bal P, Pouny I. Fitoterapia 2000;71(3):338-340.

[8] Yadav SK, Sehgal S. J Food Sci Technol; 2002;39(1): 42-46.

[9] Cutillo F, Abrosca B, Dellagreca M, Marino CD, Golino A, Previtera L, Zarrellia A. Phytochem 2003;64(8): 1381-1387.

[10] Cutillo F, D'Abrosca B, DellaGreca M, Zarrelli A. Chem Biodivers 2004 ;1(10): 1579-83.

[11] DellaGreca M, Di Marino C, Zarrelli A, D'Abrosca B. J Nat Prod 2004; 67(9): 1492-5.

[12] Dellagreca M, Previtera L, Zarrelli A. Nat Prod Res. 2005; 19(1): 87-90.

[13] Cutillo F, DellaGreca M, Gionti M, Previtera L, Zarrelli A, Phytochem Anal 2006;17(5):344-9.

Synthesis, Characterization and Biocidal Activity of Ethylene dicystein and their Metal Complexes

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ABSTRACT

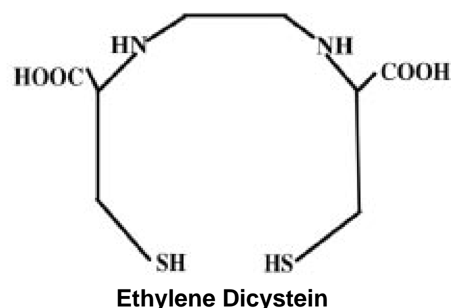
Ethylenedicystein (Ec) has two carboxylic group as well as diamine dithoil (N_2S_2) ligand system. Thus ethylene dicystein is a hexadentate system. Oxotechnetium complex and its analogue oxorhenium complex with Ec have been synthesised with slight modifications. cobalt, iron and copper complexes have been prepared in different way than oxotechnetium and oxorhenium complexes. Complexes have been structurally correleated through elemental analyses, molar conductance, FT-IR, FT-NMR, and ESR, magnetic moment, reflectance spectra and mass spectral datas. Oxotechnetium and oxorhenium complexes have been found to be pentacoordinated. Cobalt and copper complexes are square planar structure whereas iron has low spin octahedral structure. Further free ligand and their metal complexes have been screened for their antimicrobial activity and their biopotency.

Keywords: Ethylene dicystein complexes, synthesis, characterization, biocidal activity.

Introduction

The recent development in coordination chemistry of technetium and rhenium is due to the importance of the radioisotopes of these elements for diagnosis and therapy in nuclear medicine [1-3]. In addition to technetium and rhenium, copper, cobalt and iron containing coordination compounds are also of interest to nuclear medicine [4-6]. ^{99} Tc containing complex with N_2S_2 chelator has been used to characterize tumor tissues, since N_2S_2 containing complexes are stable.

In view of the importance of ethylene dicystein (Fig. 1) containing complexes with radiolabelled metal ions; the present paper discusses the synthesis, characterization and biocidal screening of complexes of oxotechnetium, oxorhenium, cobalt, copper and iron with Ec.



Ethylene Dicystein

Fig.1: Structure of Ligand

Material and Methods

All the chemicals were procured from Sigma Aldrich, U.S.A. Ethylenedicystein was synthesized as reported earlier [7] (.001 m mol) of Ec was dissolved in 4ml of N / 10 NaoH solution. In this solution (.001 m mol) of NH_4 ReO_4 / NH_4 TcO_4 in 10 ml of water has been added. Later on lithium hydroxide monohydrate (17 m mol) was dissolved in minimum amount of aqueous solution and added to solution mixture of Ec, NH_4 ReO_4 / NH_4 TcO_4 . At the end methanolic solution (5 ml) of $SnCl_2$, $2H_2O$ (.17 m mol) added dropwise under N_2 atmosphere to the solution mixture containing Ec, NH_4 ReO_4 / NH_4 TcO_4 and lithium hydroxide monohydrate. The mixture was refluxed at about 700C under N_2 atmosphere overnight. It was cooled to 00C and then filtered. After evaporation of methanol., the aquous solution of technetium complex was acidified to pH ~2 with concentrated HCl. The yellow microcrystalline needles formed within half an hour. It was dried in vacuo. Now, after evaporation of methanol from rhenium complex solution, an orange coloured aqueous solution was obtained. It was then acidified to Ph ~ 3 with concentrated HCl. The purple microcrystalline needles of Re complex formed within half an hour and dried in vacuo.

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The technetium complex obtained in form of yellow needles.

Other metal complexes were prepared by dissolving appropriate amount of respective metal chlorides in appropriate solvent and subsequently mixing them with appropriate amount of Ec solution. Solution mixture was refluxed for 5 hour at 60 – 65^{0}C under N_2 atmosphere. Solution mixture was kept in freezer for overnight and lypholized. All complexes were partially soluble or insoluble in organic solvents but soluble in NaOH. The elemental analyses and magnetic moments value of ligand and complexes were recorded at Galbraith laboratories, inc Knoville, TN 37950-1610, Atlanta. Molar conductance mesaured on Elico (CM & 2T) conductivity bridge available in the deptt. FT-IR of Ec and complexes were recorded on Perkin elmer spectrum-2000 GX, deptt. of molecular pathology, M.D. Anderson Cancer Centre. 1 H-NMR (D_2O) spectra recorded at NMR facility centre of M.D. Anderson cancer centre on Hitachi model R-600 FT-NMR using TMS as a reference. Mass spectra were recorded at the University of Texas Health Science centre (HOUSTON, TX) by fast atom bombardment on a kratos MS 50 instrument (England). EPR spectra of complex as polycrystalline were recorded on a varian E4 spectrometer using the DPPH as the g-marker. Electronic spectra has been recorded on Perkin-Elmer 137 spectrophotometer available in the deptt. The magnetic moment values of complexes recorded at National Institute for Interdisciplinary science and Technology (CSIR) Thiruvananthapuram.

Results & Discussion

On the basis of elemental analyses, complxes have been assigned composition which is shown in the table1. On the basis of elemental analyses ligand Ec has been assigned the formula $C_8 H_{16} N_2 S_2 O_4$. The molar conductance measurements of the complexes in DMSO correspond to their non-electrolyte nature except for Fe(III) complex. Therefore complexes may be formulated as [M (I) (L) O], [M (II) (L) X_2] and [M (III) (L)] respectively; (where M (I) – Tc(V); Re(V); M (II)-Fe(III); M(III)-Co(II) ; Cu(II); L-Ec and X-Cl)

FT-IR Spectra

The FT-IR spectra of ligand Ec and its respective metal complexes were recorded in KBr. The ligand exhibits νCOOH (Carboxylic group), at 1716 cm-1 . Another medium intense absorption band at 3400, 3250 and at 2550 cm-1 corresponding to ν(NH) and a ν(SH) have also been observed [8].

FT-IR of [MVOL] (M = Tc, Re; L=Ec) exhibits band between 976-980 cm-1 corresponding toνM=O band [9]. Since νM=O is singlet. it can be inferred that oxygen is attached to both metals at its trans position.Further disappearance of band near 3400, 3250 and 2550 cm-1 in these complexes is indicative of coordination through nitrogen and sulphur. Further two weak band at 2940 cm-1 have been observed in the free ligand which may be assigned to ν(H_2C- NH - C) of amine moiety stretching group vibration. The downward shift of about 30-40 cm-1 in one of ν(H_2C- NH - C) stretching vibrations show that coordination through one of the nitrogen atom of ν(H_2C- N - C) group with Tc and Re oxo complexes have taken place.

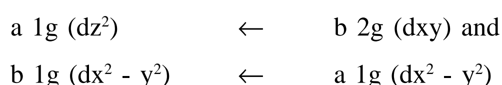
Iron complex also exhibits disappearance of band near 3400, 3250 and at 2530 cm^{-1}. This was observed in the free ligand. This is indicative of coordination through Nitrogen and Sulpher. Further iron complex exhibits negative shift of about 50 cm-1 in both (CH_2-NH-C) of amine moiety stretching vibrations, inferring coordination through both nitrogen atoms of -NH group. The ligand band shows a medium intense ir band at 2530 cm-1 due to ν(S-H) stretch. The disappearance of νSH band at 2530 cm-1 in iron complex indicates deprotonation of thiol group and consequent coordination of sulphur atom to metal ions . A band at 320 cm-1 has also observed as singlet which can be assigned to νFe-Cl band. Thus chlorine atom is at trans position.

The free ligand bands observed at 3400 and 3250 cm-1 shows downward shift by 20 cm-1 in Co-Ec and Cu-Ec complexes. This indicates coordination through amino nitrogen to metal ion . Another free ligand band at 2550 cm-1 has been found absent in these complexes; indicative of coordination through sulphur atom with copper and cobalt metal ions. However, free ligand band observed at 1716 cm-1 shows no change in oxotechenitium, oxorhenium, cobalt, iron and copper complexes of Ec, ruling out possibility of coordination through carboxylic group. In far infrared region, additional medium non-ligand bands around 390 and 270 cm-1 have been observed, which can be assigned to νM-N and νM-S vibrations respectively. [10]

Thus, on the basis of above discussion it can be concluded that in Tc and Re complexes coordination has taken place through Nitrogen , Sulphur and Oxygen. In iron complex; in addition to Nitrogen and Sulphur, coordination through chlorine has also taken place. Cobalt and copper complexes exhibit coordination through both nitrogen and sulphur.

Electronic absorption Spectra/Reflectance Spectra

Iron complex under study is dark coloured which is generally found in compounds of N, S containing ligands [11]. Because of very intense low lying charge transfer bands, obscuring d-d- transition are observed in the visible spectra of such compounds. The absorption band observed at 11500 cm-1 in iron complex can be tentatively assigned as d-d transition band. Reflectance spectra of cobalt complex under study exhibits band at 8500 cm-1 and 2100 cm-1 corresponding to transitions



a 1g (dz^2) ← b 2g (dxy) and

b 1g (dx^2 - y^2) ← a 1g (dx^2 - y^2)

Here some orbital contribution to the moment is likely to arise from eg set (dxz,dyz pair) since these are interconvertible by rotation by 900 about the Z-axis. Reflectance spectra of copper complex exhibits three absorptions at 19250 - 18620 ($\in$ - 2.3) , 22000-21500 ($\in$ = 4.2) and 23700- 23200 ($\in$ = 8.6) corresponding to 2B1g→2A1g, 2B2g and 2Eg transitions respectively. These bands are in accordance with C4V symmetry. Re and Tc complexes reflectance spectra exhibit three transitions in the region 12200-12100, 17100-17040, 23250-23150 cm-1 corresponding to the transition 2B2→2E (ν1), 2B1 (ν2), and 2A1 (ν3) respectively [12].

ESR Specrtra

ESR spectra of polycrystalline copper complex is of axial type with $g_{II} > g\perp$ (2.2051 > 2.0501) > 2.0023 and is consistent with a primarily $dx^2\text{-}y^2$ ground state in square planar structure G = (gII-2) / (g⊥- 2) which is the measurement of the exchange interaction between copper centres in a polycrystalline solid has been calculated. The reported value of G for copper complex is generally < 4 indicating the exchange interaction in solid complex. G value for copper complex in present case has been found to be 2.44. This value is indicative of strong field ligands and metal ligand bonding in the complex is covalent. Further g = 1/3 (2g⊥ + g II) which is a measurement of degree of localization of electrons on metal ion, this value comes to be nearly equal to 2.00. In case of copper complex under study, g value has been found to be 2. 153. The positive shift indicates that mixing takes place with the filled ligand orbitals.

FT-NMR Spectra

1H NMR spectra of ligand in D_2O solution shows peak at δ 2.90-2.60 ppm (multiple signal, 4H and $-CH_2$ - SH of ethylene dicystein) and at δ 3.80 ppm triplet due to coupling of adjacent (CH_2 , CH) protons of amine of ligand 1H NMR spectrum of complexes have also been recorded in D_2O. Re and Tc complexes exhibit an upward shift in amino proton peak (δ =3.70-380 ppm in free ligand and nearly 5.00-4.90 ppm in complexes) and a sharp signal at δ8.62 ppm also appeared, indicating coordination through deprotonated nitrogen atom of one of amino group . NMR signal observed in the free ligand at δ 2.90 -2.60 ppm exhibit a upward shift and two sharp signals at δ 3.10 ppm observed in rhenium and technetium complexes. This indicates coordination through both suplphur atoms of ($-CH_2$-SH). It has been further confirmed by the presence of a sharp signal at δ 7.32 ppm . In cobalt and copper complexes nmr spectra show a downward shift in amino proton peak (δ 8.61 ppm in free ligand and at 6.51 ppm in complex) and upward shift of ($-CH_2$-SH) group from δ 2.90 -2.60 ppm in free ligand to δ 3.10ppm in iron complex, indicative of coordination through one of the sulphur atom of ($-CH_2$-SH)

Magnetic Susceptibility

Room temperature magnetic moment value of both technetium and rhenium complex is in accordance with five coordinated (Table-1) structure [13]. The μeff value of iron, cobalt and copper have been measured from room temperature to liquid nitrogen temperature. The magnetic moment value of iron complex is in the range of low spin complex [14]. Low spin Fe(III) complexes with t2g5 configurations, usually have considerable orbital contributations to their magnetic moment, values. In the present case magnetic moment value of Fe^{III} comlex decreases from room temperature (2.35 M.B. - 2.00 B.M.) to liquid N_2 temperature. This shows high covalent and electron delocalization in iron comlex.

The magnetic moment value of copper complex is very close to spin only for one unpaired electron and is in the range normally observed for Cu(II) complex having an orbitally nondegenerate group state, ruling out the possibility of any metal-metal interaction The variation in the magnetic moment value of copper complex from room temperature to liquid nitrogen temperature (2.12-1.75) B.M. also reflects that there is no metal-metal bonding. Magnetic moment value of Co(II) complex is in accordance with the square planar complexes in which strong field ligands with π bond sufficiently well to compensate for the energy lost through the four rather than six co-ordination. The variation in magnetic moment of Co(II) complex from room temperature to liquid nitrogen temperature (2.65-2.2) B.M. is also in agreement with square planar structure. It is generally suggested that unpaired electron in Co(II) complex occupies the dz^2 orbital, which is responsible for its square planar structure [15].

Mass spectrum of complexes:- The mass spectrum contains peak at m / z 380.90, 467.33, 394.00, 325.22 and 327.89 for Tc, Re, Fe, Co and Cu respectively which corresponds exactly to the assigned molecular mass (Table-1)

Biocidal Screening

Antifungal activity

The antifungal activity has been evaluated against Macrophomina phaseolina and Fusarium oxysporum by the agar plate technique. Solutions of the complex in dfferent concentrations in DMF are then mixed with the medium. The linear growth of the fungus have been recorded by measuring the diameter of colony after 96h, and the percentage inhibition has been calculated as 100 (C-T) /c. The result is mentioned in Table. 2

Table -1

Colour, decomp. temperature/m.p, magnetic moment and other characterization data of legand and complexes

Legand/ Complex Comp.	Colour (Decomp/m.p.) ^{0}C	mol. mass (m/z)	Molar Conductance ohm-1 cm-1 mol-1	C	N	S	H	Cl	M	µeff. BM
Ec	Colourless 245^{0}	268	—	35.52 (35.82)	10.20 (10.44)	23.48 (23.88)	5.7 (5.97)	— —	— —	— —
TcO (Ec)	Yellow 285	380	9.89	24.98 (25.26)	7.19 (7.37)	16.55 (16.84)	3.30 (3.42)	— —	25.48 (26.03)	0.3 —
ReO (Ec)	Purple 350	467.23	10.12	20.29 (20.55)	5.91 (5.99)	13.59 (13.71)	2.59 (2.78)	— —	39.78 (39.82)	0.5 —
Fe (Ec) C_{12}	Green 250	394.08	89.88	24.10 (24.38)	6.96 (7.11)	16.10 (16.27)	3.78 (3.84)	17.85 (18.00)	13.98 (14.17)	2.35 —
Co (Ec)	Dark brown 268	325.25	11.21	29.38 (29.54)	8.48 (8.61)	19.65 (19.72)	4.25 (4.34)	— —	17.96 (18.12)	2.65 —
Cu (Ec)	Dark green 260	329.86	12.6	28.98 (29.12)	8.38 (8.49)	19.1 (19.44)	4.08 (4.27)	— —	18.99 (19.26)	2.12 —

Table 2:

Fungicidal Screening data of the ligand and Their metal complexes % inhibition after 96h (ppm)

Compound	Fusarium oxysporum			Macrophomina phaseo1ina		
	50	100	200	50	100	200
EC	41	48	51	39	51	55
TCo (Ec)	45	55	69	46	56	63
Reo (Ec)	46	56	68	45	57	64
Fe $(Ec)Cl_2$	47	59	67	48	59	65
Co (Ec)	43	53	67	49	60	66
Cu (Ec)	44	54	58	45	58	65

Antibacterial Activity: The antimicrobial screening of Ec and its complexes exhibit antimicrobial properties. It is interesting to note that the metal chelates exhibit more inhibitory effects than the parent ligands. The data has been incorporated in Table. 3

Table 3:
Antibacterial screening data of the ligand and their complexes

Compound	**Diameter of Inhibition Zone (ppm)**					
	E-coli		**K.aerogenous**		**.-campetris**	
	500	**1000**	**500**	**1000**	**500**	**1000**
Ec	10	12	8	11	8	12
TCo (Ec)	12	15	10	12	9	12
Reo (E_2c)	14	17	11	14	12	15
Fe (Ec) Cl_2	9	11	9	12	9	11
Co (Ec)	8	10	8	10	8	11
Cu (Ec)	7	9	7	9	7	9

The authors are thankful to Dr. David J. Yang Professor, Division of Experimental Diagnostic Imaging, Deptt. Of Nucler Medicine University of Texas, M.D.Anderson cancer centre Houstan, Tx, U.S.A. fo providing gift sample of chemical and his help to get elemental analysis, FT-IR, FT-NMR, mass spectral datas. Thaks to Dr. J.D. Sudha technical officer, regional research laboratory (CSIR), Thiruvanan thapuram for ESR and magnetic measurements.

References

[1] Deutsch E, Libson K, Jurission S, Lindoy L F. Prog Inorg Chem 1983; 30 : 75

[2] Deutsch E, Libson K. Comments Inorg Chem 1984; 3 : 83

[3] Clarke M J, Podbi elski L. Coord Chem. Rev 1987; 78:253

[4] Morphy J R, Parker D, Kataky R, Eaton A W M, Millican A T, Alexander R, Harrison A, walker C J. Chem Soc Perkin Trans 1990 ; 2 : 573

[5] Kovacic P, Poop W J, Ames J R., Ryan M D. Anti-cancer Drug Design 1988; 3: 205

[6] Overnell J, Good M, Vasak M. Eur J Biochem 1988; 172: 171

[7] Yang D J, Kim C G, Schechter N R, Azhadarinia A A, Yu D F. Oh C S , Bryant J L., Won J J, Kim E E, Podoloff A D. Radiology 2003; 226:465

[8] Mohan M, Agarwal A, Jha N K.Journal of Inorg Biochem 1988; 34 :41

[9] Davison A, Orvig C, Trops H S, Sohn M, Depamphilis B V, Jones A G. Inorg Chem 1980; 19:1988

[10] Mohan M, Sharma P, Kumar M, Jha N K. Inorg Chim Acta; 1986:;125:9

[11] Padhye S, Kauffman G.B. Coord Chem Rev 1985 ; 63 : 127

[12] Hathaway B J, Bradley J N, Gillard R D, Hudson R F. Eassy in Chemistry 2nd Edition Academic Press Newyork 1971:61

[13] Rouchias G. Chem Review 1974; 5-75: 531

[14] Lewis J, Williams J P. Modern coordination chemistry 2nd Edition Inter science New York : 470

[15] Cotton F A, Wilkinson G, Murillo A C and Bochman M. Advance Inorganic chemistry 6th Edition John wiley & sons, New York 2004:790

Studies on Extracellular *L-glutaminase* production by Halophilic Aspergillus sp.

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ABSTRACT

L-Glutaminase is an enzyme which is used in the treatment of leukemia and also extensively used in the food industry. In the present investigation, glutaminase production pattern was studied under submerged fermentation using isolated halophilic Aspergillus sp. The analysis of enzyme production pattern revealed that the maximum production was noticed at pH 6.0, rpm 140, incubation temperature 28^0C, incubation period for 72 hours, 2% inoculum concentration and 2% glutamine concentration. Variation of carbon source supplementation improved the productivity from 22.97 to 27.64 U/ml with 1% (w/v) lactose. Addition of $NaNO_3$ to the fermentation medium improved the glutaminase production. Under the above optimized environment glutaminase production was improved by 136% with this microbial isolate.

Keywords: glutaminase, leukemia, glutamic acid, submerged, halophilic.

Introduction

L-Glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) catalyzes the hydrolysis of L-glutamine to L-glutamic acid and ammonia. This is an essential enzyme for synthesis of various nitrogenous metabolic intermediates. Glutaminase also acts as a catabolic repressor in microorganisms [1]. It is also present in the mammalian tissues and break downs the glutamine into glutamic acid and ammonia [2]. Although L-glutaminase has been reported from many bacterial genera, members of the *Enterobacteriaceae* family have been best characterized. Among bacteria *E.coli* glutaminases have been studied in much detail. Some examples of bacterial strains producing L-glutaminase include *E.coli, Pseudomonas* species like *P.aeroginosa, P.aureofaciens, P.aurantiaca and P.fluorescens; Acinetobacter* sps; *Bacillus* sps; *Proteus morganii; Proteus vulgaris; Xantomonas juglandis; Erwinia carotovora, E.aroideae, Serratia marcescens, Enterobacter cloacae, Klebsiella aerogens and Aerobacter aerogenes.* Among yeast species *Hansenula, Rhodotorula, Candida scotii, Cryptococcus albidus, Cryptococcus laurentii, Candida utilis, C.nodaensis, Torulopsis candida, Zygosachharomyces rouxii*. Among fungi *Aspergillus oryzae, A.sojae, Tilachlidium humicola,Verticillium althoasei* etc, have been reported to produce glutaminase.

E.coli is the only one organism used for industrial applications. Thus seemingly enormous scope exists for the search for potential strains that could produce L-glutaminase in high yields and with novel properties under economically viable bioprocesses. Wade et al, Nelson et al and Imada et al compiled more comprehensive lists of glutaminase producing organisms [3-5].

Literature shows that *Beauveria* sps., could produce extracellular L-Glutaminase even in the absence of an enzyme inducer like L-glutamine or any additional amino acid, when sea water was used in the medium [11]. Considering the potential in vitro applications of glutaminase, the extracellular nature of these enzymes is of much significance from the view point of direct attack upon raw substrate materials and due to the ease of purification. In this paper, we report the production of extracellular L-glutaminase by halophilic fungi Aspergillus sp. under submerged fermentation. The present study envisages optimizing the process and nutritional factors for L-glutaminase production by isolated microorganism.

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Materials and Methods

Micro organism and cultural condition

The Aspergillus culture used in this study was isolated from marine water of the Bay of Bengal along the Visakhapatnam coast. The medium was composed of KH_2PO_4 1 g, $MgSO_4$ 0.5 g, $CaCl_2$ 0.1 g, $NaNO_3$ 0.1 g, Trisodium citrate 0.1 g, NaCl 10.0 g, L-glutamine 10.0 g, Dextrose 5.0 g, Sea water 1lit and pH 6.0 After inoculation with a 2% (v/v) conidial suspension, incubated at 27 °C on a rotary shaker at 120 rpm. After 48 hours cultivation the mycelia was separated from the broth by centrifugation and the supernatant was used for assaying enzyme activity.

Estimation of glutaminase activity:

Glutaminase was assayed according to the method of Imada et al.where the liberated ammonia due to the action of enzyme was estimated using Nessler's reagent.

Optimization of process parameters for L-glutaminase production

The medium described above was taken as a basal medium and the different process parameters including pH (4 to 9); temperature (26 to 36 °C); rpm (80-180); inoculum concentration (0.5-3.0 % v/v); Glutamine concentration (0.5-30% w/v); additional nitrogen sources (0.5 % w/v) viz. peptone, yeast extract, malt extract, ammonium sulphate, meat extract and $NaNO_3$; additional carbon sources (0.5% w/v) viz. lactose, glucose, galactose, starch, maltose and sucrose were optimized independently. Finally the time course of production was evaluated under the optimized conditions. All experiments were conducted in triplicate and the mean values are reported.

Results and Discussion:

Any biotechnological product production whether it involves recombinant organisms or by conventional fermentation processes should be economically viable. Optimization of production parameters contributes significantly to the commercial viability of any bioprocess [6]. Hydrogen ion concentration of medium plays vital role in metabolism of any organism and each organism has its optimum pH where growth of selected microbial strain and its cellular metabolism occurs at optimum levels compared to other conditions [7] Hence, the influence of medium pH on the production of the glutaminase by isolated microbial strain has been investigated by inoculating the strain in the medium having pH range of 4.0 to 9.0 and analyzing the glutaminase production pattern regularly during the fermentation. The data revealed that this microbial strain effectively produces the enzyme in the pH range of 5.0 to 8.0 with maximum production at pH 6.0 indicating its acidic nature (Fig 1). Higher or lower medium pH resulted in lowering of production. Glutaminases are generally active at an alkaline pH. While optimal activities of Glutaminase A and B from *Pseudomonas aeruginosa* were at alkaline pH of 7.5-9.0 and 8.5 respectively [8-10]. Glutaminase from *Pseudomonas* sp, was reported to be active over a broad range of pH 5-9 with an optimum near pH 7.

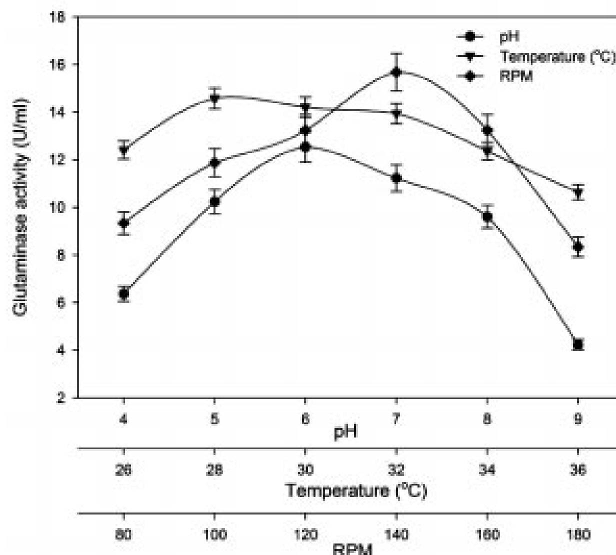

Fig 1: Influence of medium pH, Temperature and RPM on glutaminase production by isolated microbial strain.

The influence of temperature was studied by carrying out the fermentations between 26 – 36 °C with the interval of 2°C. The results were presented in Fig 1. Maximum glutaminase production was noticed at 28°C and deviation from this temperature resulted in reduction of enzyme production.

The amount of aeration has a profound effect on the rate of growth which in turn influences enzyme production. Hence, the influence of rpm on glutaminase production was investigated using this microbial strain. Enzyme production increased with increase in rpm during fermentation until 140 rpm and further increase results in decrease of glutaminase production (Fig 1.)

Since microbial productivity is directly proportional to biomass, the role of inoculum level on glutaminase production with this strain was studied. High yield of enzyme was obtained when inoculum concentration was 2% (v/v) and 48 hrs of age was used (Fig 2). It was interesting to note that increase of inoculum level up to 2% resulted in increased glutaminase production and further increase resulted in decreased production. Such inoculum concentration dependent product formation variations were reported in other microbial strains [9].

The concentration of inducers plays a pivotal role in the production of extracellular enzymes. Glutamine acts as an inducer for the production of the glutaminase. In order to find out the appropriate glutamine concentration for optimum glutaminase production the glutamine concentration was varied from 0.5 to 3.5 % in the medium. It was observed that 2% glutamine concentration (Fig 2) gives an increase of 30% in the amount of glutaminase produced. Above or

below 2% glutamine concentration decreases the glutaminase production.

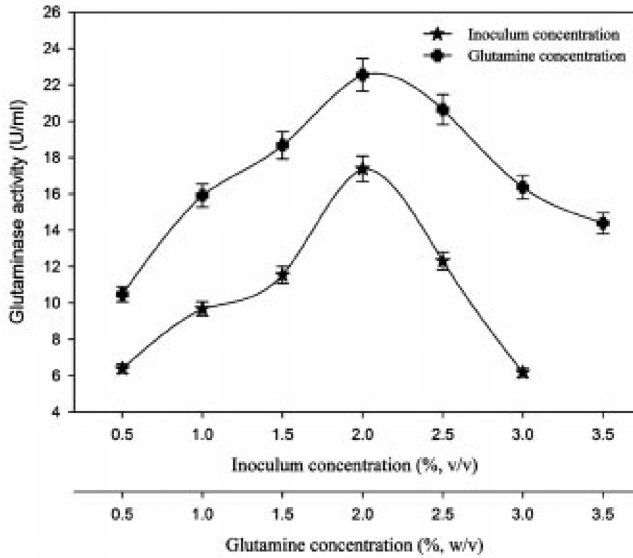

Fig 2: Influence of inoculum and glutamine concentration on glutaminase production by isolated microbial strain.

Fermentation processes are raw material cost intensive and the profitability is greatly dependent on the product yield per unit substrate consumed. In order to reduce costs, industrial processes use organic nitrogen substrates such as corn steep liquor and yeast extract. Thus, although the stoichiometric analysis is the first logical step in process development, it is often difficult to achieve due to the ill-defined nature of the medium. In order to develop an effective medium, the role of different additional carbon (Fig 3) and nitrogen (Fig 4) sources were evaluated for their influence on glutaminase production by this microbial strain. Of the various carbon sources that had been screened, lactose was found to be the best and sucrose was the least supporting carbon source for enzyme production (Fig 3 insert). Our results are similar to earlier studies on carbon source utilization which showed that the carbon source has a significant effect on growth and product formation. Glutaminase production was studied at different concentrations of lactose and the results indicated that supplementation of 1% lactose into the medium resulted in maximum enzyme production (27.64 U/ml) and an increase or decrease in lactose concentration resulted in reduced production of glutaminase (Fig 3 insert). Among different nitrogen sources studied, $NaNO_3$ enhanced glutaminase production (Fig 4).

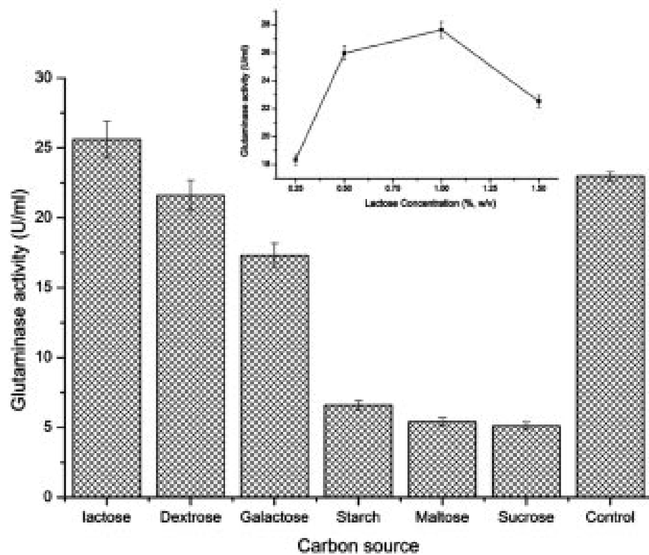

Fig 3: Effect of different carbon sources on production of glutaminase by isolated microbial strain; Effect of lactose concentration on glutaminase activity.

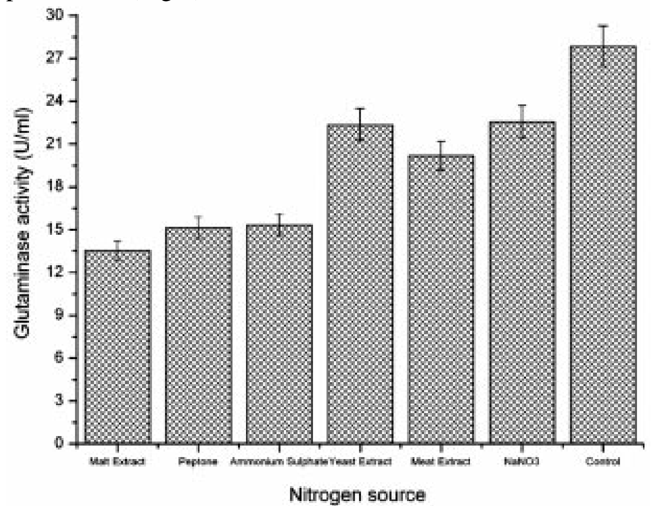

Fig 4: Effect of different nitrogen sources on glutaminase production by isolated microbial strain.

The fermentation profile indicated that maximum L-glutaminase production (29.64U/ml) occurred at 72 h of incubation (Fig 5).

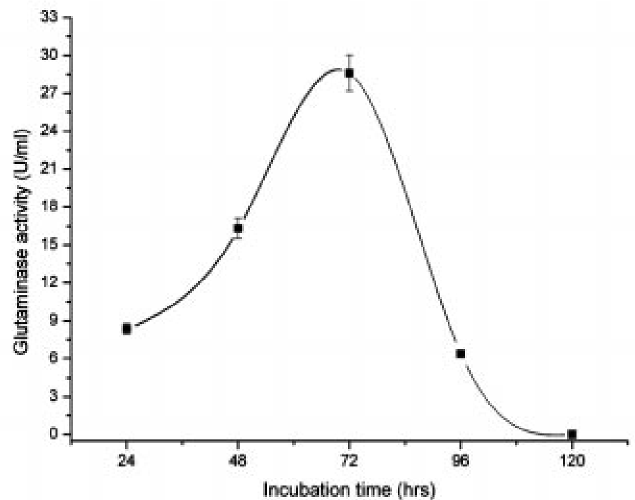

Fig 5: Effect incubation time on production of glutaminase by isolated microbial strain.

Further, the maximal L-glutaminase yield (29.64 U/ml) recorded with this fungus is at an appreciable level when compared to earlier reports for fungi (Yano et al. 1988) and bacteria (Renu & Chandrasekaran 1992; Prabhu & Chandrasekaran 1997). The present study indicates scope for the use of Aspergillus sp. as an ideal organism for the industrial production of extracellular L-glutaminase.

Conclusion

The potential of isolated microbial strain for glutaminase production was investigated under different growth and medium conditions. Maximum production was noticed at pH 6.0, at 28°C, 140 rpm, with 2% inoculum, 2% glutamine concentration, 1% lactose as carbon source and 0.5 % $NaNO_3$ at incubation period of 72 hours. Under optimized environment glutaminase production was improved from12.54 to 29.61 U/ml (136%) with this microbial isolate.

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References

[1] Calderon J, Huerta-Saquero A, Du Pont G, Duran S. Biochim. Biophys. Acta 1999; 1444: 451–456.

[2] Klein M, Kaltwasser H, Jahns T. FEMS Microbiol. Lett 2002;206: 63–67.

[3] Wade HE, Robinson HK, Philips BW. J Gen Microbiol 1971;69:299.

[4] Nelson GEN, Petrson RE, Ciegler A. Dev Ind Microbiol 1974;169: 385.

[5] Imada A, Igarasi S, Nakahama K, Isono M. J Gen Microbiol 1973;76:85.

[6] Nissen TL, Kielland-Brandt MC, Nielsen J, Villadsen J. Met Eng 2000; 2(1): 69–77.

[7] Vara AG, Hochkoepple A, Nielsen J, Villadsen J. Biotechnol Bioeng 2002; 77(5): 589–598.

[8] Prakasham RS, Subba Rao Ch, Sarma PN. Bior Technol 2006;97(13): 1449-1454.

[9] Nandakumar R, Yoshimune K, Wakayama M, Moriguchi M. J Mol Catal B: Enz 2003; 23: 87–100.

[10] Prakasham RS, Subba Rao Ch, Sreenivas Rao R, Sarma PN. Biotechnol Prog 2005;21(5):1380-1388.

[11] Keerthi TR, Suresh PV, Sabu A, Rajeevkumar S, Chandrasekaran M. World J Microbiol Biotechnol 1999;15: 751-752.

Synthesis, characterization and anti-microbial screening of novel thiazolidino-fused compounds.

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ABSTRACT

4-Thiazolidinones (III a-e) which are hetarylsubstituted at the 2-position were prepared by the reaction of mercapto acetic acid with aldimines (II a-e) which were prepared by the condensation of 4-(1*H*-benzimidazol-2-yl) benzenamine(I) with different substituted aryl aldehyde. After their benzylidene derivatives (IV a-e) were obtained, Pyrazolinothiazolidine (V a-e) were synthesized by using phenyl hydrazine in the presence of sodium acetate. All the synthesized compounds were characterized by spectral data and screened for their antimicrobial activity. Mention the compounds activity when compared with standard drugs.

Keywords: Benzimidazole, Thiazolidine-4-one, benzylidene derivatives, pyrazolinothiazolidine and anti-microbial activity.

Introduction

Aldimines have been generally used as substrates in the formation of a large number of industrial compounds *via* cycloaddition, ring closure, replacement reactions etc [1, 2]. In addition, the aldimines of heterocyclic compound, which are widely used in the production of pharmaceuticals, have taken an important place among the compounds of biological interest because of the conjugation and the groups that they contain within their molecules. Furthermore, most of the 4-thiazolidinones and their benzylidene derivatives display a large variety of activities such as antibiotic, diuretic, organoleptic, tuberculostatic, antileukemik and antiparasitical [3,4]. To our knowledge, little is known on fused thiazolidines that to possess these activities [5]. Moreover, little attention has been directed to the behavior of this class of compounds toward phenylhydrazine under varied conditions to determine their cyclization reactions for possible biological activities of new compounds [6, 7].

For a long time imines have been used successfully in the synthesis of nitrogen containing heterocycles[8]. As part of our ongoing project aimed at the discovery of bioactive 4-thiazolidinones we employed the Schiff bases, II **a-e** towards their synthesis. These azomethines have been obtained by the reaction of 2(4-amino phenyl) benzimidazole I with different substituted aromatic aldehyde in refluxing dry ethanol or dry benzene[9]. First, we obtained the new 3-(4-(benzimidazol-2yl)phenyl)-2-substituted aryl-4-thiazolidinones, III **a-e** in good yields by refluxing equimolar amounts of the imines II **a-e** and thioglycolic acids in DMF. Then compounds III **a-e** reacted with benzaldehyde in the presence of sodium acetate to afford benzylidene derivatives IV **a-e**. These were condensed with phenylhydrazine in glacial acetic acid in the presence of sodium acetate, and subsequent cyclization to give 2-substituted phenyl-6,7-diphenyl-3(4-(benzimidazol-2yl)phenyl)-pyrazolino-(3,4-d)thiazolidines V **a-e** (Scheme). The structures of the new compounds were firmly established on the basis of their IR, ^{1}H-NMR analysis and screened for their antimicrobial activity.

Materials and Methods

Experimental

Melting points were determined in open glass and are uncorrected. The purity of the compounds was ascertained by TLC on silica gel-G plate. Characterizations of synthesized compounds were done by spectral studies. IR spectra were taken in KBr on a THERMONICOLET NEXUS-670 Spectrophotometer. ^{1}H NMR spectra were recorded on AVANCE-300MH$_Z$ Spectrophotometer in DMSO-d_6 with TMS as internal standard. The chemical shift values are in delta (ppm). Physical data and antimicrobial activities of synthesized compounds were recorded in Table-1 & Table-2.

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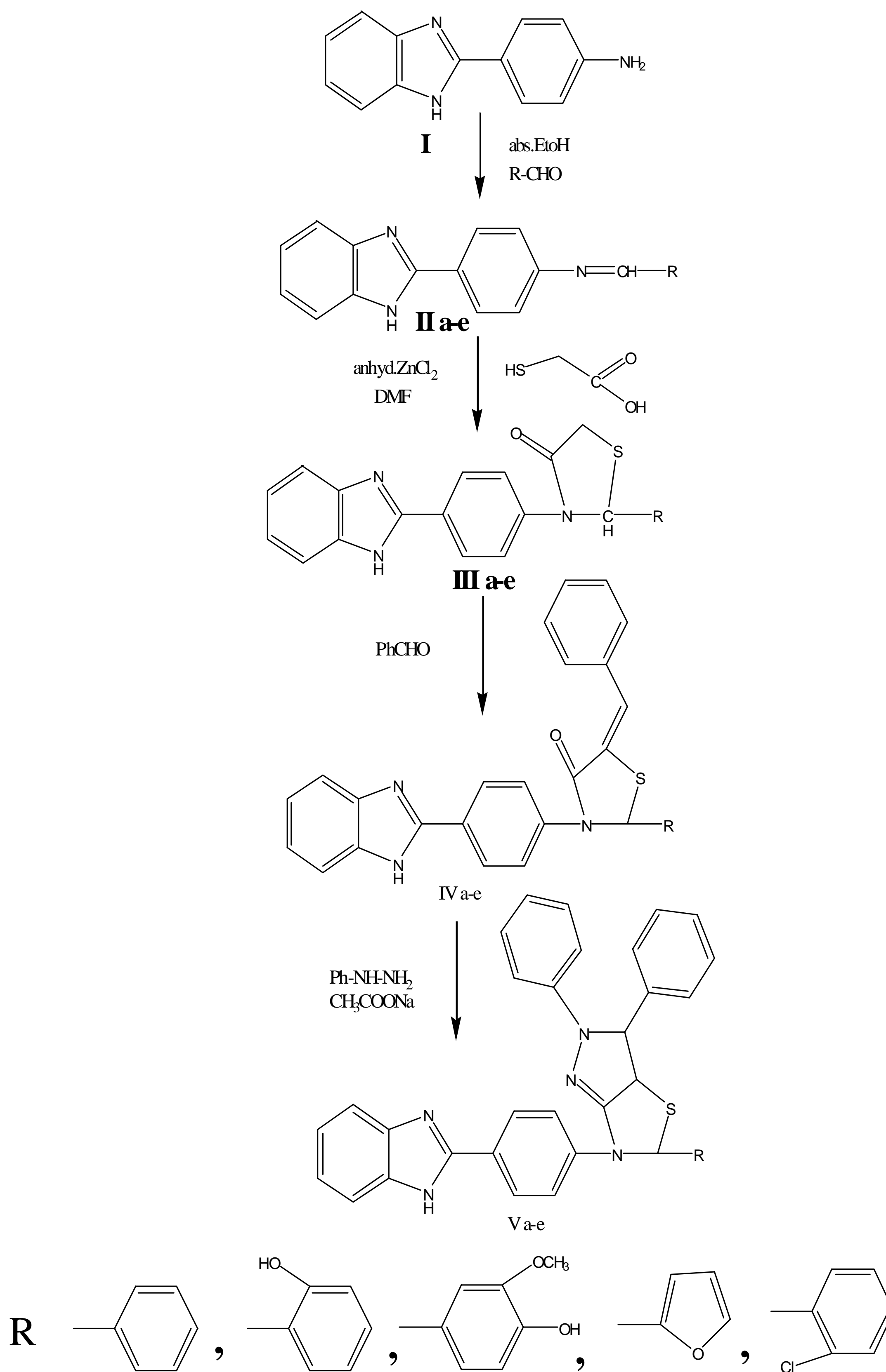

Table – 1
Physical data of Compounds

Compound	R	M.P (° C)	Yield (%)	Molecular Formula
V a	Phenyl	198 – 200	74	$C_{35}H_{27}N_5S$
V b	2-hydroxyphenyl	234 – 237	68	$C_{35}H_{27}N_5OS$
V c	4-hydroxy,3-methoxy phenyl	280	71	$C_{36}H_{29}N_5O_2S$
V d	Furan	228 – 230	64	$C_{33}H_{25}N_5OS$
V e	2 – Chloro Phenyl	244 – 246	70	$C_{35}H_{26}N_5SCl$

Table – 2
Antimicrobial activity data

Compounds (50¼g/ml)	Antibacterial activity Zone of Inhibition in (mm)			Antifungal activity Zone of Inhibition in (mm)	
	Bacillus coli ATCC11778	*Staphylococcus aureus* ATCC9144	*Escherichia coli mutant* ATCC25922	*Candida albicans* ATCC2091	*Aspergillus niger* ATCC9029
V a	22	23	21	21	19
V b	25	24	23	22	18
V c	26	25	25	23	20
V d	23	23	22	20	19
V e	20	21	21	19	17
Standard	33	29	31	33	28
Control DMF	–	–	–	–	–

Preparation of I

o-Phenylenediamine dihydrochloride (0.1mol) and p-amino benzoic acid (0.1mol) were mixed with sufficient quantity of polyphosphoric acid to give a paste, the mixture was heated slowly to 230°C and stirred at this temperature for 4 hrs. The resultant mixture was cooled to room temperature and poured in a thin stream into a large volume of $NaHCO_3$ solution and allowed to settle. The solid thus obtained was filtered and recrystallized from methanol. Yield was 68.0%, m.p 238°C.

Preparation of II a-e

Equimolar (0.01mol) quantity of compound I and substituted aromatic aldehyde dissolved in 50ml of absolute ethanol and added few drops of acetic acid then heated on steam bath for 2 hrs. After standing for 24hrs at room temperature the product was filtered, dried and recrystallized from methanol. The obtained compound was characterized by ^{1}H NMR spectral data.

II a: ^{1}H NMR (DMSO-d_6): 7.2-7.9 (m, Ar-H, benzimidazole-H), 8.7 (s, 1H, N=CH), 5.2 (s, benzimidazole-NH).

II b: ^{1}H NMR (DMSO-d_6): 6.7-7.9 (m, Ar-H, benzimidazole-H), 5.9 (s, phenolic-OH), 8.5 (s, 1H, N=CH), 5.3 (s, benzimidazole-NH).

II c: ^{1}H NMR (DMSO-d_6): 6.3-7.7 (m, Ar-H, benzimidazole-H), 8.6 (s, 1H, N=CH), 5.8 (s, phenolic-OH), 3.4 (s, O-CH_3), 5.1 (s, benzimidazole-NH).

II d: ^{1}H NMR (DMSO-d_6): 6.6-7.9 (m, benzimidazole-H, furan-H), 5.1 (s, benzimidazole-NH), 8.8 (s, 1H, N=CH).

II e: ^{1}H NMR (DMSO-d_6): 7.2-7.9 (m, Ar-H, benzimidazole-H), 5.2(s, benzimidazole-NH), 8.6 (s, 1H, N=CH).

Preparation of III a-e

A mixture of II a-e (0.01 mol) and thioglycollic acid (0.01 mol) in DMF (30ml) containing a pinch of anhydrous zinc chloride was refluxed for 6 hrs. The reaction mixture was cooled and poured over crushed ice. The solid thus obtained washed with sodium bicarbonate solution and recrystallized from glacial acetic acid. The obtained compound was characterized by IR, ^{1}H NMR spectral data.

III a: IR (KBr): 3324.8 cm^{-1} (NH), 2924.6 cm^{-1} (Ar-H), 1601.68 cm^{-1} (C=O), 695.4 cm^{-1} (C-S-C)

^{1}H NMR (DMSO-d_6):7.0-7.9 (m, Ar-H, benzimidazole-

H), 5.2 (s, benzimidazole-NH), 6.1(s, N-CH-S), 3.5 (s, S-CH_2-).

III b: IR (KBr): 3256.6 cm^{-1} (NH), 3059.78 cm^{-1} (Ar-H), 1642 cm^{-1} (C=O), 3454 cm^{-1} (OH), 694.4 cm^{-1} (C-S-C)

^{1}H NMR (DMSO-d_6): 6.7-7.8 (m, Ar-H, benzimidazole-H), 5.1 (s, benzimidazole-NH), 5.8 (s, N-CH-S), 3.7(s, S-CH_2-), 5.3 (s, Phenolic-OH).

III c: IR (KBr): 3058.93cm^{-1} (NH), 2925.05 cm^{-1} (Ar-H), 1655.08 cm^{-1} (C=O), 3245cm^{-1} (-OH), 693.08 cm^{-1} (C-S-C)

^{1}H NMR (DMSO-d_6): 6.8-7.9 (m, Ar-H, benzimidazole-H), 5.1 (s, benzimidazole-NH), 5.9 (s, N-CH-S), 3.3 (s, S-CH_2-), 4.2 (s, O-CH_3), 5.4 (s, Phenolic-OH).

III d: IR (KBr): 3375.06 cm^{-1} (NH), 2921.33 cm^{-1} (Ar-H), 1638.93 cm^{-1} (C=O), 636.86 cm^{-1} (C-S-C)

^{1}H NMR (DMSO-d_6): 6.2-7.9 (m, furan-H, benzimidazole-H), 5.2(s,benzimidazole-NH), 6.3 (s, N-CH-S), 3.5 (s,S-CH_2-).

III e: IR (KBr): 3375.26 cm^{-1} (NH), 2922.19 cm^{-1} (Ar-H), 1638.78 cm^{-1} (C=O), 694.09 cm^{-1} (C-S-C)

^{1}H NMR (DMSO-d_6): 7.2-7.9 (m, Ar-H, benzimidazole-H,), 5.2 (s, benzimidazole-NH). 5.9 (s,N-CH-S), 3.6 (s,S-CH_2-).

Preparation of IV a-e

A mixture of III a-e (0.005mol), benzaldehyde (0.005mol) and anhydrous sodium acetate (0.005mol) in glacial acetic acid (50ml) was refluxed on a heating mantle for 3 hrs. The reaction mixture was concentrated, cooled and poured into ice-cold water. The solid thus obtained was filtered, washed with water and recrystallized from ethanol to give crystals yield-65%. The obtained compound was characterized by IR, ^{1}H NMR spectral data.

IV a: IR (KBr): 3323.8 cm^{-1} (NH), 2925.6 cm^{-1} (Ar-H), 1642.68 cm^{-1} (C=O), 1670 cm^{-1} (C=CH).

^{1}H NMR (DMSO-d_6):6.9-7.2(m, Ar-H, benzimidazole-H), 7.6 (s, benzylidene-H), 5.2 (s,benzimidazole-NH),5.8 (s,N-CH-S).

IV b: IR (KBr): 3258.6 cm^{-1} (NH), 3049.78 cm^{-1} (Ar-H), 1642 cm^{-1} (C=O), 1674.96 cm^{-1} (C=CH), 3454 cm^{-1} (OH)

^{1}H NMR (DMSO-d_6): 6.5-7.5 (m, Ar-H, benzimidazole-H), 7.8 (s, benzylidene-H), 5.1 (s,benzimidazole-NH),5.7 (s, N-CH-S), 5.5 (s, Phenolic-OH).

IV c: IR (KBr): 3059.93cm^{-1} (NH), 2927.05 cm^{-1} (Ar-H), 1657.08 cm^{-1} (C=O), 1671.95cm^{-1} (C=CH), 3255cm^{-1} (-OH)

^{1}H NMR (DMSO-d_6): 6.2-7.4 (m, Ar-H, benzimidazole-H), 7.8 (s, benzylidene-H), 5.31 (s, benzimidazole-NH), 5.9 (s, N-CH-S), 4.0 (s, O-CH_3), 5.6 (s, Phenolic-OH).

IV d: IR (KBr): 3376.06 cm^{-1} (NH), 2926.33 cm^{-1} (Ar-H), 1648.93 cm^{-1} (C=O), 1673.66 cm^{-1} (C=CH)

^{1}H NMR (DMSO-d_6): 6.2-7.6 (m, furan-H, benzimidazole-H), 7.9 (s, benzylidene-H), 5.21 (s,benzimidazole-NH),6.0 (s, N-CH-S).

IV e: IR (KBr): 3378.26 cm^{-1} (NH), 2923.19 cm^{-1} (Ar-H), 1640.78 cm^{-1} (C=O), 1674.28 cm^{-1} (C=CH).

^{1}H NMR (DMSO-d_6): 6.6-7.2 (m, Ar-H, benzimidazole-H), 7.6 (s, benzylidene-H), 5.2 (s, benzimidazole-NH). 5.86 (s,N-CH-S).

Preparation of V a-e

A mixture of IV a-e (0.0025mol), phenyl hydrazine (0.005mol) and anhydrous sodium acetate (0.005mol) in glacial acetic acid (50ml) was refluxed on a heating mantle for 5 hrs. The reaction mixture was concentrated, cooled and poured into ice cold water. The solid thus obtained was filtered, washed with water and recrystallized from ethanol to give crystals yield-62%. The obtained compound was characterized by IR, ^{1}H NMR spectral data.

V a: 1R (KBr): 3324.79 cm^{-1} (NH), 2924.62 cm^{-1} (Ar-H), 1601.68 cm^{-1} (C=N).

^{1}H NMR (DMSO-d_6):7.0-8.28 (m, Ar-H, benzimidazole-H, pyrrole-H), 5.2 (s,benzimidazole-NH),5.66 (s,N-CH-S), 3.68 (d,1H,8-CH),4.10 (d,1H,7-CH).

V b: 1R (KBr): 3256.6 cm^{-1} (NH), 3059.78 cm^{-1} (Ar-H), 1601.08 cm^{-1} (C=N).

^{1}H NMR (DMSO-d_6): 7.2-8.1 (m, Ar-H, benzimidazole-H, pyrrole-H), 5.7 (s,benzimidazole-NH),6.8 (s, N-CH-S), 5.3 (s, Phenolic-OH), 3.58 (d,1H,8-CH),3.90 (d,1H,7-CH).

V c: 1R (KBr): 3058.93cm^{-1} (NH), 2925.05 cm^{-1} (Ar-H), 1601.95 cm^{-1} (C=N).

^{1}H NMR (DMSO-d_6): 7.3-8.1 (m, Ar-H, benzimidazole-H, pyrrole-H), 5.4 (s, benzimidazole-NH),6.7 (s, N-CH-S),2.5 (s,O-CH_3), 5.6 (s, Phenolic-OH), 3.4 (d,1H,8-CH),3.80 (d,1H,7-CH).

V d: 1R (KBr): 3375.06 cm^{-1} (NH), 2921.33 cm^{-1} (Ar-H), 1603.66 cm^{-1} (C=N).

^{1}H NMR (DMSO-d_6): 7.0-7.9 (m, furan-H, benzimidazole-H, pyrrole-H), 6.35 (s,benzimidazole-NH),6.4 (s, N-CH-S), 3.78 (d,1H,8-CH),3.90 (d,1H,7-CH)..

V e: 1R (KBr): 3375.26 cm^{-1} (NH), 2922.19 cm^{-1} (Ar-H), 1604.28 cm^{-1} (C=N).

^{1}H NMR (DMSO-d_6): 7.0-7.9 (m, Ar-H, benzimidazole-H, pyrrole- H), 6.5(s, benzimidazole-NH). 6.76 (s,N-CH-S), 3.46 (d,1H,8-CH),3.93 (d,1H,7-CH).

Antibacterial activity

All the compounds were tested for their antibacterial activity against *Bacillus cereus* ATCC11778, *Staphylococcus aureous*ATCC9144 (Gram +ve), *Escherichia*

coli ATCC25922 (Gram -ve) using disc diffusion method [10, 11]. DMF was run as a control and test was performed at different concentrations using a solvent DMF. Ciprofloxacin used as a standard drug. All the pyrazolinothiazolidine derivatives (V a-e) showed antibacterial activity.

Anti-fungal activity

All the compounds were tested for their antifungal activity against *Candida albicans* ATCC 2091 and *Aspergillus niger* ATCC 9029 by disc diffusion method [10, 11]. DMF was run as a control and test was performed at different concentrations using a solvent DMF. Ketoconazole used as a standard drug. All the compounds (V a-e) showed antifungal activity.

Results and Discussion

The structures of the new compounds were firmly established on the basis of their IR, ^{1}H-NMR analysis. Regarding compounds V a-e showed two doublets at 3.4 ppm and 3.9 ppm respectively for the proton at 3'a and 3' position corroborated the cyclic structure and cis configuration. In the IR spectra of the thiazolidinones, the characteristic C=O bands appeared in the region of 1680-1640 cm^{-1}. The strong sharp bands at 1620-1610cm^{-1} corresponding to initial azomethines were absent, which was the most characteristic evidence of the cyclocondensation. Regarding compounds V a-e amide carbonyl band was absent which clearly confirmed that a cyclocondensation with phenyl hydrazine had taken place. In general, all th compounds showed antibacterial and antifungal activity. The maximum activity was obtained when R was substituted by a hydroxyl, methoxy group in the phenyl ring of thiazolidine nucleus (26mm). The thiazolidine derivatives having chlorine atom showed minimum activity (17mm). Rest of the compounds showed moderate activity. It was found that the compounds possessing electron releasing groups considerably enhanced the antimicrobial activity when compared to the electron withdrawing substituents on the phenyl ring. In summary, we have synthesized a new series of potentially bioactive substituted pyrazole fused with thiazolidinyl moieties.

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References

[1] Brown AD, Colvin EV. Tetrahedron Lett 1991; 32:5187.

[2] Burwood DA, Gallucci J, Hart DJ. J Org Chem 1985; 50: 5120.

[3] Brown FC. Chem Rev 1961; 61: 463.

[4] Raasch MS. J Heterocyclic Chem 1974; 11: 587.

[5] Singh VP, Upadyay GS, Singh H. Asian J Chem Rev 1992; 3: 12.

[6] Kouznetsov V, Öcal N, Turgut Z, Zubkov F, Kaban S, Varlamov AV. Monatsh Chem 1998; 129:671.

[7] Ocal N, Yolacan C, Kaban S, Vargas YLM, Kouznetsov V. J Heterocyclic Chem 2001; 38: 233.

[8] Kouznetsov VV, Prostakov NS. Khim Geterotsikl Soed 1990; 1: 5 Chem. Abstr 1990; 113: 40347b.

[9] Mhamadou A, Barbier JP, Hugel RP. Polyhedron 1992; 11: 2697.

[10] Collee J.G, Duguid J.P, Fraster A.G, Marimion B.P, Makie. Practical Medical Microbiology 13th ed., Churchill living stone, London 1989: 163-165

[11] Ananthanarayanan R, Panikr JCK. Text Book of Microbiology 5th ed., Orient Longman Ltd.1987: 620-621

Screening of antibiotic producing *actinomycetes* from terrestrial soil and river sediments of *Cauvery* basin, Karnataka.

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ABSTRACT

This study screened actinomycetes isolated from terrestrial soil and river sediment isolated of Cauvery basin, Karnataka state for antimicrobial activity. A total of 54 actinomycetes isolates were obtained from the various terrestrial soil and river sediments samples collected and were tested for antagonistic activity against *E.coli* (NCIM-2563)*, S. aurias* (NCIM-2492), *C.utilis* (NCIM-3055) *and A.niger* (NCIM-1222). Results indicated that 14 out of 54 isolates were active against at least one of the test microorganism, 13 isolates were active against atleast one of the test bacteria and 4 were active against one of the test fungi. It was noted that the terrestrial site was the richest source of the antibiotic – producing actinomycetes where approximately 40% of isolates were antibacterial and 4% were antifungal. Selected bioactive isolates were chosen for further screened against other strains of the test microorganisms during secondary screening. Resulting mean diameter of inhibition zones revealed isolates SII-45, SIV-05 and RI-12 are the most potent of all remaining isolates with a minimum inhibitory microbial concentrations 20%(MIC) for SII-45 and 15%(MIC) for SIV-05 and RI-12. Cultural and morphological characterization classified them under the genus *Streptomyces*. It can be recommended therefore terrestrial soil and river sediment samples from cauvery basin, karnataka state be further investigated for anti-tumor and antibiotic producing actinomycetes. The number of actinomycetes isolated with persistent activity suggests Cauvery basin of Karnataka state may be India's potential source of novel antibiotics.

Keywords: antimicrobial, *actinomycetes*, cauvery basin, minimum inhibitory concentraton.

Introduction

Actinomycetes are considered to be the most widely distributed group of micro-organisms in nature which primarily inhabit the soil [1]. Many of them are prolific producers of various kinds of bioactive compounds. They provide over two-third of the naturally occurring antibiotics discovered and continued to be a major source of novel and useful compounds

Most of actinomycetes are known to have the capacity to synthesize bioactive secondary metabolites which include enzymes, herbicides, pesticides, and antibiotics. Almost 80% of the worlds antibiotic are known to come from actinomycetes, mostly from the genera *streptomyces* and *macromonospora* [2].

Actinomycetes consist of an extensive and diverse group of gram-positive, aerobic and mycelial bacteria that play an important ecological role in soil cycles. Current efforts are focused on the development and massive application of selective methods for the isolation of members of the genus actinomycetes and related genera.

According to the World Health Organization, over prescription and improper use of antibiotics has led to the development of resistance by many pathogens. Clinically-important bacteria, such as *staphylococcus aureus*, are becoming resistant to commonly used antibiotics. At present, new resistant strains are being emerged more quickly, while the rate of discovery of new antibiotics is slowing down. Because of this, many scientists have focused on screening programs of microorganisms, primarily of actinomycetes, for the production of antibiotics [1].

The present work reports isolation, characterization and screening of potent actinomycetes.

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Materials and Methods

Sample collection and processing

Soil samples and sediments were obtained from twelve different locations each throughout a river site, a terrestrial site in cauvery basin, mysore and madikeri district, Karnataka, India. From different locations seven terrestrial soil samples and five river sediment samples of 5g each were collected from 10 to 15cm below the surface.

All the samples were placed in small pre-labeled sterile plastic bags which tightly sealed. The temperature and pH of the soil were determined. Sediment samples were air dried for one week. For isolation studies serial dilutions were made up to 10^{-6}.

Isolation of actinomycetes from terrestrial soil and river sediments:

About 0.1ml of each dilution was surface plated onto Yeast Malt Extract Agar (YMA) supplemented with cyclohexamide (50 µg/ml) and tetracycline (20µg/ml). These were incubated for one week at room temperature. After incubation, actinomycetes isolates were distinguished from other microbial colonies by characteristics such as tough, leathery colonies which are partially submerged into the agar [3]. Each isolate was then coded based on the sample of origin. Terrestrial isolates were given the code Si#, where Si which stands for terrestrial isolate and the # for the number of the isolate. River isolates were given the code Ri# respectively. Pure cultures were inoculated in 10 ml of yeast malt extract broth (YMB) and incubated at room temperature for 24 to 48 hours in a rotary shaker (200 rpm) prior to screening

Antibacterial screening of actinomycetes isolates

Two stages of antibacterial screening were done. All isolates were subjected to primary screening while secondary screening was only performed for the upper 50% of the total actinomycetes isolates tested that showed inhibition during the primary screening.

Preliminary antimicrobial screening

Actinomycetes isolates were screened for antimicrobial property using the Agar Well Method on Mueller-Hinton Agar plates. Pure cultures of the test bacteria (*Escherichia coli, **Staphylococcus aureus**, Candida **utilis** and Aspergillus **niger***) were obtained in nutrient broth from the national collection of industrial microorganism, national chemical laboratory Pune, India. Suspensions of test organisms were adjusted to 10^8 cfu/ml (Mac Farland standard 0.5) for use in the primary and the secondary screenings. The YMB tubes containing the actinomycetes isolates were centrifuged for 15 minutes at 7000 rpm to separate the pellet and the supernatant. Each resulting supernatant was decanted onto a sterilized test tube. Inoculation of 100 ml of the test microorganism in culture broth in an MHA plate was done by spread-plating. The antimicrobial activity was determined by the Agar Well method [2], distinguishing the pellet from the supernatant. YMB was used as the negative control. Fifty microliters of each were then loaded into their respective wells. The plates were sealed and incubated at 37°C for 18 to 24 hours, and then observed. The diameters of the zones of complete inhibition were measured in millimeters to the nearest hundredths using a calibrated Vernier caliper. Four millimeters was subtracted from the obtained diameters of complete inhibition. The isolates exhibiting zones of inhibition against any test bacteria were chosen. Triplicates of each were made, and the diameters of the inhibition zones were again measured by the millimeter using a calibrated Vernier caliper.

Secondary antimicrobial screening

The most potent isolates were noted for each test microorganism. Based on the mean diameter of inhibition zones, they were subjected to secondary screening of antimicrobial assay now using clinical and pathogenic strains of bacteria and fungi which they inhibited

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the actinomycetes isolate having the largest inhibition zone measured based from the secondary screening was determined by broth dilution test using varying concentration of the actinomycetes suspension (0, 5, 10, 15, 20, 25, 25, 50, 75, 100%) prepared using YMB.

Characterization of the Most Potent Actinomycetes isolate

The most potent actinomycetes isolate was characterized by macroscopic and microscopic morphological methods. The macroscopic method was done by colony characterization on YMA-color of colony and presence of pigmentation was recorded. The microscopic characterization was done by cover slip culture method observed after 3 day. The presence or absence of aerial and substrate mycelium, spore formation, fragmentation of the vegetative or substrate mycelium were observed.

Result and Discussion

Isolation of actinomycetes from terrestrial soil and river sediments:

Terrestrial soil samples were obtained form seven locations and river sediments from five locations in each of the two sites that are terrestrial and river.

In each area, temperature and pH were obtained to determine appropriate culture conditions. Temperature of sediments ranged from 22-32^{0}c while the pH varied from 7.5 to 9.2. These two readings for the substrates were within ranges that are said to be optimum for actinomycetes growth and survival [4].

The two sediment types of the sampling area yielded 54 actinomycetes isolates. A total of 30 actinomycetes are isolated from terrestrial sites, while 24 were recovered from the river sites [5] showed that actinomycetes were less

common in river sediments relative to terrestrial soils, another study by Goodfellow and Haynes [6] suggested that actinomycetes represent only a small component of the total bacterial population in river sediments. It was observed that most of the isolates were obtained from terrestrial origin. Terrestrial soils have the main reservoir of actinomycetes [7], they comprise the large part of the microbial population of the soil [8].

Antimicrobial screening of actinomycetes isolates:

Preliminary antibacterial screening

Thirteen of the 54 actinomycetes isolates showed antagonistic property in at least one of the two test bacteria. Meanwhile, four (three from the terrestrial site and one from the river site) inhibited at least one of the test fungus. Based on the findings, about 40 % of the 30 terrestrial isolates and 4 % of 24 river isolates were antibacterial whereas only two actinomycetes isolates were found active against *C. utilis*. Five actinomycetes isolates were found active against *E. coli* and twelve against *S. aureus*. Three replicates were made for the 13 actinomycetes isolates active against the test bacterial microorganisms. Their mean diameter of inhibition zones were computed and recorded. Four millimeters, the diameter of the well, was subtracted from the obtained mean diameter. From the five actinomycetes isolates which inhibited *E. coli*, only one (SII-45) was chosen to advance to the secondary screening. Three of the five isolates showed activity against *E. coli* with both the supernatant and pellet. The other two, namely, SIII-42, SII-24, had an inhibitory effect only with the use of pellet (Figure 1).

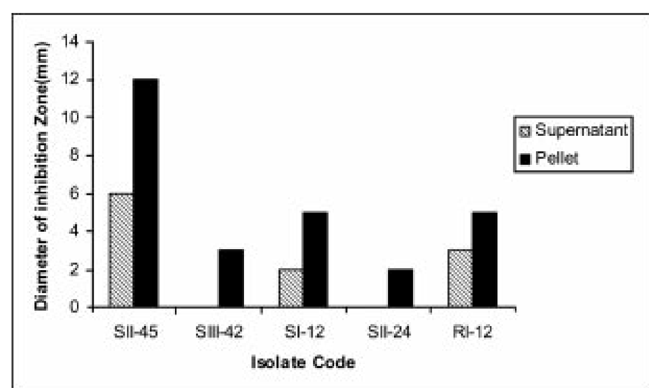

Fig.1: Activity of Actinomycetes Isolates against *E.coli.*

Both supernatant and pellets of nine of the 10 isolates were found to inhibit the growth of *S. aureus*. Isolates SIII-26 has recorded inhibitions for the pellets only. Results also revealed that pellets were more active for most isolates (Figure 2).

Only the pellet of SVI-14 was active on *C. utilis* which also gave the greatest mean diameter of inhibition zones (Figure 3).

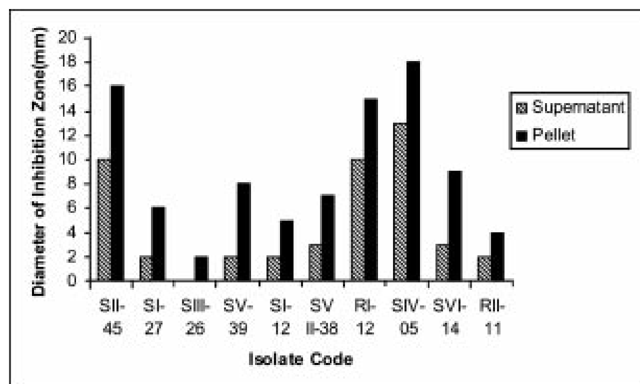

Fig.2: Activity of Actinomycetes Isolates against *Staphylococcus aureus*

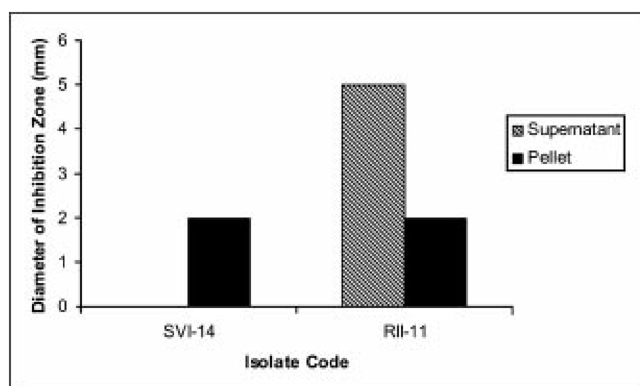

Fig.3: Activity of Actinomycetes Isolates Against *C.utilis*

Likewise, only two isolates were active against *Aspergillus niger* with isolate SIV-32 &SV-18 having the greatest diameter of inhibition zone for both pellets and supernatant (Figure 4).

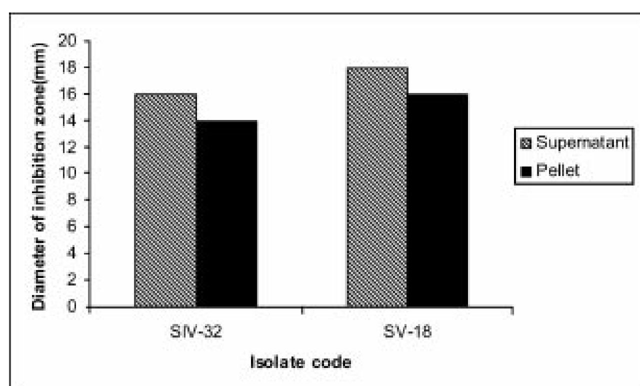

Fig.4: Activity of Actinomycetes Isolates Against *A.niger.*

Results of the primary screening suggested that the actinomycetes found in cauvery basin exhibited antimicrobial activity against the two test bacteria, namely *E. coli and S. aureus*. Most except one active actinomycetes were isolated from terrestrial sediments which suggests that this proportion of antibiotic producers and where they were found were influenced by the chemical soil characteristics

of the area since production of secondary metabolites by actinomycetes is directly affected by its environment. He demonstrated in his study that 78% of Actinomadura isolates obtained from reportedly rich mangrove soils exhibited antimicrobial activity, while only 14% were active from poor, actinomycetes-rich tropical soils [9].

Results further revealed that cauvery basin isolates were found to be more active against gram-positive bacteria (*S. aureus*) than gram-negative bacteria (*E. coli*) which corroborated with previous observations [2, 1, and 10] likewise observed that activities against gram-negative bacteria were less frequent than against gram-positive bacteria. Results also showed that most of the active isolates were more active against bacteria than fungi. This agreed with the findings of the study done by Nedialkova and Naidenova (2004) [11]. The study showed that isolated active actinomycetes strains Antarctica with a broader spectrum of antibacterial activity and only limited antifungal activity against various strains of yeasts and phytopathogenic fungi.

Although, various studies have also shown the above mentioned pattern of antimicrobial activity, this is not always the case [12]. Comparison of antibacterial and antifungal activities of isolated actinomycetes strains has not been the focus of majority of the studies dealing with antimicrobials since most fungi require longer generation time compared to bacteria. Many antibacterial agents inhibit steps important for the formation of peptidoglycan, a very important cell wall component. On the other hand, most antifungal compounds, target either the formation or the function of ergosterol, an important component of the fungal cell membrane [13]. However, this dominance of actinomycetes strains with antibacterial activity in cauvery basin, karnataka might be a reflection of the soil ecology. Secondary metabolites (antibiotics) are produced by some actinomycetes in order to overcome competition with other soil microorganisms by killing or destroying them. From this,

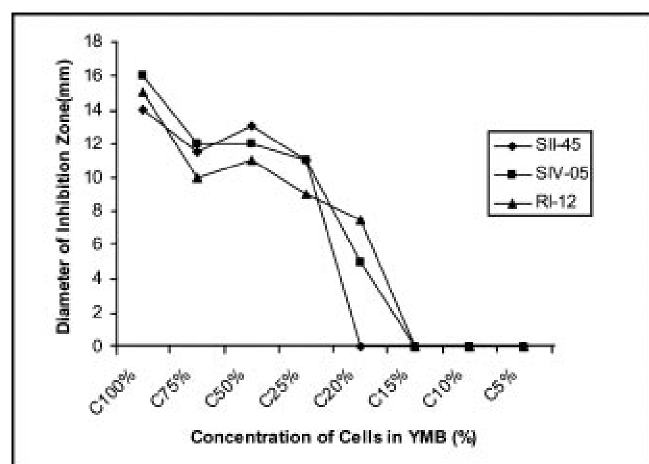

Fig.5: Minimum inhibitory concentration (MIC) of the different concentration of SIV-32 supernatant.

we could infer that the sediments from which the actinomycetes isolates were obtained have a rich bacterial population which competes with them. As a means of adaptation, they produce secondary metabolites which are harmful to these bacteria.

Secondary Antimicrobial Screening.

Out of the 13 actinomycetes isolates subjected to the preliminary antibacterial screening, only five were subjected to secondary antibacterial screening to verify their consistent activity on other strains of the same test microorganisms. It can be noticed in Table 3 that there were varied observed activities as compared to the primary screening. No isolate was found to inhibit the clinical strain of *E. coli* and only one isolate was found to be active against the clinical strain of *S. aureus*. Isolate SVI-14 supernatant, compared to its pellet, showed an improved activity against *S. aureus* relative to the primary screening results but still exhibited larger inhibition zone than its supernatant. Screening of the active actinomycetes isolates against clinical strains of *E. coli and S. aureus* showed considerable changes in their antimicrobial activities. Those isolates active against non pathogenic *E. coli,* except for SVI-14 which was active against non-pathogenic *S. aureus*, exhibited no activity against the respective clinical strains of test bacteria which may be attributable to the fact that these clinical strains of bacteria may possess characteristics that differ or are absent from the non-pathogenic strains. Pathogenic strains of *E. coli* may possess specific virulence determinants (toxins and adhesions, etc.) encoded by monocistronic genes, plasmids, or pathogenicity islands as well as plasmids that code for drug resistance, which may partially account for the ineffectiveness of most of the antibiotics produced by the isolate[14]. Pathogenicity of *S. aureus*, on the other hand, is mainly caused by several virulence factors, one of which is its inherent and acquired resistance to antimicrobial agents [15].

Only *A. niger* AN-20 was used as the test fungus for the secondary screening since primary screening revealed that no isolate were found to be active against the yeast test organism. Results showed that both isolate SIV-32 and SV-18 that inhibited *A. niger* showed activity in the secondary screening. Improved activity was shown by the SV-18 supernatant against a cauvery basin strain of *A. niger*. Isolate SV1-32 had the largest mean diameter of inhibition zones (Table 4).

Bacteria and fungi usually share a common substrate. Spatial proximity has allowed for the emergence of synergistic or antagonistic interactions between them, probably as a result of competition. Filamentous fungi, including *Aspergillus niger*, are very proficient at secreting native proteins. Some of these enzymes have the ability to counteract another organism, i.e., actinomycetes, and their activity. Different strains of *A. niger* produce different types and amounts of enzymes. It could then be inferred that

Table 3

Mean diameters of inhibition zones (mm) of test microorganisms by bioactive actinomycetes isolates in primary (1^0) and secondary (2^0) screening

Isolate code	Mean diameter of zones of inhibition(mm)							
	Escherichia coli				*Staphylococcus aureus*			
	supernatant		Pellet		Supernatant		Pellet	
	1^0	2^0	1^0	2^0	1^0	2^0	1^0	2^0
SII-45	6	0	12	0	10	0	16	0
RI-12	3	0	5	0	10	0	15	0
SIV-05	0	0	0	0	13	0	18	0
SVI-14	0	0	0	0	3	5	9	7
RII-11	0	0	0	0	2	0	4	0

Table 4

Mean diameters of zones of inhibition (mm) of *A.niger* by bioactive actinomycetes isolates in primary (1^0) and secondary (2^0) screening

Isolate code	Mean diameter of zones of inhibition (mm)			
	Supernatant		Pellet	
	1^0	2^0	1^0	2^0
SIV-32	16	12	14	17
SV-18	18	11	16	15

different strains of the fungus, when exposed to a certain antifungal actinomycetes isolate, may vary in their responses. That is, they may either remain unaffected or be inhibited. In this study, *A. niger* differs in certain ways from the cauvery basin strain. Using another strain of *A. niger* was necessary to test the consistency of the activity of both isolates SIV-32 and SV-18 on the specific species of mold. In another observation, in all active isolates obtained from the terrestrial site, inhibition zones appear around the wells containing either pellet or supernatant. Moreover, among all the active terrestrial isolates, the measurements of inhibition zones around wells containing the pellet were larger than those around wells containing the supernatant. Nkanga (1978) [16] stated that the antimicrobial activity of freshly isolated actinomycetes in liquid culture is low because of their initially low production of antibiotics. In order to detect an inhibition, the antibiotic produced must be concentrated. The actinomycetes isolates were grown in 10 ml YMB which may have been diluted in the broth. Hence, they would have been more diluted in the supernatant than in the pellet, and consequently cause a larger inhibition zone in the latter.

Surprisingly, a larger inhibition zone of *S. aureus* was observed in the supernatant of RII-11 as compared to its pellet. This may be due to the nature of the antibiotic produced by this isolate. According to Waksman (1959) [17], pigments play various roles in the growth and survival of actinomycetes. One such role is for defense against foreign cells, they being referred to as antibiotic pigments. Production of more than one antimicrobial compound has been proven in several studies. They mentioned that in addition to the calcium-dependent antibiotic (CDA), *Streptomyces coelicolor* produces three other known antibiotics, namely, actinorhodin, methylonomycin and undecylprodigiosin, and one novel polyketide, mutactin. In this study, intracellularly- and extracellularly- produced antibiotics were accounted through the use of pellet and supernatant, respectively.

Determination of the Minimum Inhibitory Concentration (MIC)

Isolate SII-45, SIV-05 & RI-12 were chosen to be subjected for minimum inhibitory concentration (MIC) assay since it exhibited the larger zone of inhibition after the two screenings. Data shows that the MIC of isolate SII-45, SIV-05, RI-12 falls between 15-20%. It can be inferred then that to exhibit antimicrobial activity against *S. aureus*, a microbial suspension having at least 20% actinomycetes cells is needed. Pandey[2] mentioned about the MIC is not a

constant for a given agent, since it is influenced by a number of factors. These factors include the nature of the test organism used, the inoculum size, and the composition of the culture medium, the incubation time, and aeration. Hence, to obtain more accurate results for MIC than what have been reported in this study, it would be better if the bioactive compound will be isolated by extraction techniques in future studies.

Characterization of the most potent actinomycetes isolate

Both macroscopic and microscopic methods were employed to describe SIV-05, SII-45 and RI-12. According to Waksman (1961) [18], such color and form(table5) are exhibited by both colonies of Streptomyces. However the color of the growth and the form of the colony could not serve as basis for finding out the genus to which actinomycetes isolate belongs to. Hence, its morphological characters must serve as the primary basis of characterization. One distinguishing morphological character isolates SiV-05, SII-45 and RI-12 commonly possesses abundant reddish brown to reddish pink aerial mycelium[19]. A well developed substrate mycelium partly penetrates the medium and the formation of smooth, straight or spiny and hairy spores with occasional hooks at the sporophore tips are the two characteristics that may well qualify it as a species of Streptomyces (Krassilinikov, Kuster, Preobrazhenskaya et al) [20-23]. The formation of dark brown pigment that dissolves into the medium has also been mentioned by Waksman(1961) [18] to be a possible characteristics of the genus although not exclusive. After growing from broth, isolates SIV-05, SII-45 & RI-12 were plated on yeast extract malt extract agar and was seen to exhibit yellowish brown, brown pale yellow colonies respectively.

However subsequent transfer of isolates shows brownish yellow colonies. The forms of colonies were described to be round, flat too convex with concentric circled colonies with powdery and spreading edges. It was also observed that very pale brownish pigmentation was produced on the medium. Also, both well defined reddish brown to reddish pink aerial mycelium and well developed substrate mycelium were observed on the medium.28^0 Studies made supporting the anti tumor antibiotic activity of streptomyces have further strengthened the claim that isolates SIV-05, SII-45 & RI-12 does belongs to said genus.

SIV-05 is nearer to *S.pucitis* but differed from the references culture in the following respects sprophore morphology, color of substrate mycelium and its fragmentation cell wall composition, tyrosinase reaction, starch hydrolysis, nitrate reduction. In view of this large number of significant differences it is proper to consider that our isolate SIV-05 is a new species and it is designated as *S.kanurubidis.*

SII-45 is closer to *S.fumicarius* but differed in following aspects: sporophore morphology, inability to hydrolysis of gelatin, nitrate reduction, absence of antifungal activity. In view of these significant differences it is proper to consider out isolate as a new species and is designated as *S. cauverensis*.

Few differences between our isolate RI-12 and the reference culture *S.xanthociducus* could be noticed like

Table 5

Cultural and morphological characteristics of SIV-05, SII-45 & RI-12

Character	Observations		
	S IV-05	**S II-45**	**RI-12**
Temperature	28^0C	28^0C	29^0C
pH	7.0	7.0	7.2
Colony color	Brown	Yellow brown	Pale brown
Nurture of colony	Round, convex colonies and spreading edges	Round, flat to convex compact	Round, convex colonies with concentric circles & powdery spreading edges
Pigmentation in medium	Present(pale brown)	Absent	Present(pale yellow)
Aerial mycelium	Abundant (reddish brown)	Present (dark pink)	Present(bluish grey)
Substrate mycelium	Present	Present	present
sporophores	Long and straight with occasional hooks	Straight to waves & occasionally as open hooks	Short coiled spirals

sporophore morphology, absence of antifungal activity, sodium chloride tolerance. In view of these minor differences, it is proposed to consider our isolate RI-12 as a variant of *S.xanthocidicus* and it is designated as *S.kanurensis*.

Conclusion

Based on the screening results, it has been shown that terrestrial soil of Caurvery basin, Karnataka State, posses antitumor antibiotic producing actinomycetes and may be tapped as one of the Indians potential source of novel antibiotics.

References

[1] Oskay M., Tamer AU, Azeri C. Afr J Biotechnol 2004.

[2] Pandey B, Ghimire P, Agrawal VP. Studies on the antibacterial activity of actinomycetes isolated from the Khumbu region of Mt. Everest. A paper presented in the International Conference on the Great Himalayas: Climate, Health, Ecology, Management and Conservation, Kathmandu. January 12-15, 2004. Organized by Kathmandu University and the Aquatic Ecosystem Health and Management Society, Canada

[3] Jensen PR, Dwight R, Fenical W. App Env Microbiol 1991; 57:1102-1108

[4] Catedrilla CM. Isolation and identification of some antibiotic-producing actinomycetes from Laguna de Bay sediments. (Undergraduate Thesis, University of the Philippines Los BaÒos, 1991)

[5] Goodfellow M & Williams ST. Ann Rev Microbiol 1983;37:189-216.

[6] Goodfellow M & Haynes JA. Actinomycetes in marine sediments. In Biological, biochemical and biomedical aspects of actinomycetes. Ortiz-Ortiz, L., Bojalil, L.F. and Yakoleff, V. (Eds.) Orlando: Academic Press 1984, pp-453-472.

[7] Grein A & Meyers SP. J Bacteriol 1958; 76:457-463.

[8] Athalye M, Lacey J, Goodfellow M. J App Bact 1981;51: 289-297.

[9] Srivibool R. Bur Sci J 1998; 6: 23-33.

[10] Basilio A, Gonz·lez I, Vicente MF, Gorrochategui J, Cabello A, Gonz·lez A, Genilloud O. J App Microbiol 2003; 95(4):814-823.

[11] Nedialkova D & Naidenova M. J Culture Collections 2004; 4:29-35.

[12] Kokare CR, Mahadik KR, Kadam SS, Chopade BA. Cur Sci 2004; 86: 593-597

[13] http://cmr.asm.org/cgi/content/full/12/4/501

[14] http://textbookof bacteriology.net/e.coli.html

[15] Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T, Tenover FC. J Ant Chem 1997; 42: 315-320

[16] Nkanga EJ, Hagedorn C. Antimicrobial Agents and Chemotherapy 1978; 14(1):51-59.

[17] Waksman SA. The actinomycetes: Nature, Occurrence, and Activities. 1959 Baltimore: The Williams and Wilkins Company

[18] Waksman SA. The Actinomycetes: Classification, Identification and Descriptions of genera and species. 1961 Baltimore: The Williams and Wilkins Company

[19] Lechevalier. Bergeyís manual of systematic bacteriology, Volume IV, Ninth Edition. 1994 Baltimore: The Williams and Wilkins Company

[20] Krassilenikov NA. 'Determination bacteria and actinomycetes" Acad. Sci., USSR Institute of microbiology, Moscow, 1949.

[21] Kuster,E., Zent. Bakteriol. Parasitenk. Abst.II 1955; 108:376.

[22] Preobrazhenskaya TP, Kudrina ES, Sveshinikova MA, Maksimova TS, Mikrobiologiya 1959; 28:623.

[23] Preobrazhenskaya TP, Kudrina ES, Maksimova TS, Sveshnikova MA, Boyanskaya RV, Mikrobiologiya 1960; 29:51.

A new analytical method development and validation for the simultaneous estimation of ramipril, atorvastatin and clopidogrel in capsule dosage form by RP-HPLC

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ABSTRACT

The working conditions for the high pressure liquid Chromatography method was established for Ramipril, Atorvastatin and Clopidogrel and then applied on pharmaceutical dosage forms. A rapid sensitive of Chromarographic separation was accomplished using hypersil BDS C18 (250×4.6 mm, 5μ) analytical column with mobile phase consisting of potassium phosphate buffer (pH 2.8), acetonitrile and methanol (50:45:5 v/v). The eluent were detected by UV absorbance at 215 nm. The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantification and solution stability. The proposed method can be used for the estimation of these drugs in combined pharmaceutical dosage forms.

Keywords: RP-HPLC, Ramipril, Atorvastatin and Clopidogrel.

Introduction

Ramipril is chemically designated as (2s,3as,6as)-1-[(s)-2-((s)1-1(ethoxycarbonyl)- 3-phenyl propyl) amino] octahydro cyclopenta (b) pyrrole-2-carboxylic acid. Ramipril is an angiotensin-converting enzyme (ACE) inhibitor. An inactive prodrug, ramipril is converted to ramipril in the liver and is used to treat hypertension, heart failure, to reduce proteinuria and renal disease in patients with nephropathies and to prevent stroke, myocardial infarction, and cardiac death in high-risk patients [1]. Atorvastatin calcium is chemically [R-(R*, R*)-2-(4-fluorophenyl- β,α-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamine)carbonyl]-1H-pyrrol-1-heptanoic acid, calcium salt (2:1) trihydrate. Atorvastatin, a selective, competitive HMG-CoA reductase inhibitor, is used to lower cholesterol and triglycerides in patients with hypercholesterolemia [2]. Clopidogrel bi sulphate is chemically methyl(+) –(s)-(2chlorophenyl)-6,7 –dihydrothieno [3,2-c] pyridine-5(4H)-acetate sulphate[3]. Survey of literature for Ramipril, Atorvastatin and Clopidogrel revealed several methods based on HPLC [4,5], GC/MS [6] for its determination in human plasma and in pharmaceutical formulations. No single method was reported for the estimation in combined pharmaceutical dosage form. The present RP-HPLC method was validated following the ICH guidelines [7,8].

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Experimental

Reagents and chemicals

Acetonitrile HPLC grade was procured from E. Merck Ltd, Mumbai. Glacial acetic acid and potassium dihydrogen phosphate AR grade were procured from Qualigens Fine Chemicals, Mumbai. Water HPLC grade was obtained from a Milli-QRO water purification system. Reference standards Atorvastatin calcium and Clopidogrel bi sulphate were procured from Biocon Limited, Bangalore and Ramipril was procured from Aarthi Drugs in Mumbai.

Apparatus and Chromatographic conditions:

The HPLC system consisted of a separation module (Water Alliance 2695) and Photo Diode Array (PDA) detector all from waters. (Water's Corporation, (USA) An isocratic elution was performed on a Hypersil BDS C18 column (250 mm x 4.6 mm, 5μ).

The mobile phase was a degassed and filtered (0.45 µ, Millipore) mixture of phosphate buffer solution (PH 2.8), aceto nitrile and methanol (50:45:5v/v).

Injection volumes were 20µl and flow rate was established on 2.0 ml/min. The UV detector wavelength was set at 215 nm for the determination of all Ramipril, Atorvastatin, Clopidogrel. Quantification was achieved by peak-area-ratio method with reference to the standards.

Preparation of standard solutions:

About 50 mg of Ramipril, 108 mg of Atorvastatin calcium, 978 mg of Clopidogrel bi sulphate weighed individually and transferred into a 50 ml standard flask. The powder was dissolved by sonication using sufficient amount of diluent and then made upto the mark with the same so as to give a concentration of 100 µg/ml of Ramipril, 200 µg/ml of Atorvastatin and 1500 µg/ml of Clopidogrel.

Preparation of sample solution:

Twenty capsules were weighed. A quantity of powder equivalent to 50 mg of Ramipril, 108 mg of Atorvastatin calcium, 978 mg of Clopidogrel bi sulphate weighed individually and transferred into a 50 ml standard flask. The powder was dissolved by sonication using sufficient amount of diluent and then made up to the mark with the same so as to give a concentration of 100 µg/ml of Ramipril, 200 µg/ml of Atorvastatin and 1500 µg/ml of Clopidogrel.

Assay method:

With the optimized Chromatographic conditions, a steady baseline was recorded, the mixed standard solution of 20 µl was injected and the chromatogram was recorded. From the chromatogram the retention time of Ramipril, Atorvastatin and Clopidogrel was found to be 2.936, 8.037 and 12.727 respectively. The procedure was repeated for the sample solution obtained from the formulation. The amount of Ramipril, Atorvastatin and Clopidogrel present in each capsule was calculated by comparing the peak area of the sample and standard solution. The amount of the drugs were calculated and tabulated in Table No:1

Results and Discussion

The HPLC procedure was optimized with a view to develop precise and stable assay method. Various mobile phase systems were prepared and used to provide an appropriate chromatographic separation, but the proposed mobile phase comprising of potassium phosphate buffer PH2.8, acetonitrile and methanol in the ratio (50:45:5v/v) gave a better resolution and sensitivity. The detection was carried out by using UV-visible PDA detector at 215 nm. Amongest the several flow rates tested (0.5 - 2.0 ml), the flow rate of 2.0 ml/min was the best for all the drugs with respect to location and resolution of analytical peaks. The retention time of Ramipril, Atorvastatin and Clopidogrel was found to be 2.936, 8.037 and 12.727 respectively. Fig.1 and 2 are those of quantitative estimation report of standard and sample. The asymmetry factor or the tailing factor of Ramipril, Atorvastatin and Clopidogrel bi sulphate was found to be 0.5612, 0.497 and 0.6944 respectively, which indicates symmetrical nature of the peak. The percentage of individual drugs found in formulations were calculated and presented in Table No.1. The results of analysis shows that the amounts of drugs were in good agreement with the label claim of the formulations.

Method Validation:

Accuracy and Precision:

The accuracy of the method was determined by recovery experiments. The recovery studies were carried out three times and the percentage recovery and standard deviation of the percentage recovery were calculated and presented in Table No. 2. From the data obtained added recoveries of standard drugs were found to be accurate.

The Precision of the method was demonstrated by system precision and method precision studies. In the system precision studies, six replicate injections of the working standard solution prepared as per the proposed method and chromatograms were recorded and shown in Fig.3. Standard deviation and relative standard deviation was calculated and presented in Table No.3. From the data obtained, the developed RP-HPLC method was found to be precise.

Table No.1

Results of Analysis of Formulation

Sl. No.	Capsule sample	Label claim (mg/capsule)	*Peak Area	*Amount Present (mg/capsule)	*PercentageLabel claim(%w/w)
1.	Ramipril	5	1514341.667	4.978	99.80
2.	Atorvastatin	10	134662.8	19.99	99.98
3.	Clopidogrel	75	2891748.8	74.96	99.98

** Each Value is mean of six readings.*

Table No. 2

Accuracy report for Ramipril, Atorvastatin and Clopidogrel

S.No	Sample	Recovery	Area obtained	Average area	Amount Recovered in mg	%Recovery
1	Ramipril	80	1212986 1213567 1214953	1213835	3.94	79.9
		100	1513378 1513972 1514187	1513846	4.9702	99.815
		120	1816374 1815573 1814610	1815519	5.98	119.68
2	Atorvastatin	80	4107115 4106493 4105934	4106514	7.92	79.2
		100	5134876 5134681 5134902	5134819.6	9.865	98.651
		120	6166389 6165923 6167341	16166551	11.91	119.1
3	Clopidogrel	80	26084358 26083714 26084976	26084349.3	59.91	79.88
		100	32456317 32457921 32455863	32456700.3	74.432	99.242
		120	39074682 39073611 39076244	39074875.6	89.78	119.71

Table No.3

System Precision report

Parameters	Area of Ramipril	Area of Atorvastatin	Area of Clopidogrel
Trail 1	1513913	5134004	32468499
Trail 2	1513282	5134472	32459821
Trail 3	1514862	5133453	32458179
Trail 4	1514364	5136876	32459685
Trail 5	1514781	5135201	32465892
Trail 6	1516048	5135654	32459164
Average	1514541.66	5134943.33	32461873.33
Standard Deviation	942.60	1235.49	4243.55
% Relative Standard Deviation	0.0622	0.0240	0.0130

Linearity and Range:

The linearity of the method was determined at five concentration levels ranging from 80-120 µg/ml for Ramipril, Atorvastatin and Clopidogrel using pure drugs (Table No.4). The slope and intercept value for calibration curve was y=15130.37960x+588.47024 (R2=0.9999) for Ramipril; y =25367.06600x-1560.537292 (R2 =0.9999) for Atorvastatin and y =63983.88x+16474.95 (R2 =0.9999) for Clopidogrel. The results show that an excellent correlation of concentration of drugs within the concentration of drugs and within the concentration range indicated above. (Fig.No. 3-5)

Limit of Detection (LOD) and Limit of Quantification (LOQ):

The Limit of Detection (LOD) and Limit of Quantification (LOQ) of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method. The LOD is the measurable response (signal to noise ratio 3). The LOD for Ramipril, Atorvastatin and

Table No.4.
Linearity report

Sl. No	Ramipril		Atorvastatin		Clopidogrel	
	Conc.µg/ml	Avg peak area	Conc.µg/ml	Avg peak area	Conc. µg/ml	Avg peak area
1	80.16	1212126.66	162.09	4107111	1563.84	26084495
2	90.18	1364487.93	182.35	4622141	1778.4	29308458
3	100.2	1525091.28	202.62	5134843	1976	32586547
4	110.22	1665371.49	222.88	5658722	2173.6	35822132
5	120.24	1816774.68	243.14	6166687	2371.2	39075409

Clopidogrel was found to be 0.6 µg/ml, 0.21 µg/ml and 24.84 µg/ml respectively. The LOQ is the measurable response. The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10). The LOQ was 1.96 µg/ml, 0.48 µg/ml and 26.72 µg/ml for Ramipril, Atorvastatin and Clopidogrel respectively Table No.5.

Table No.5
System suitability report

Parameter	Ramipril	Atorvastatin	Clopidogrel
Tailing factor	0.5612	0.497	0.6944
Number of theoretical Plates	964	6869	7491
Capacity factor	1.919	7.053	11.73
Limit of Detection	0.6µg/ml	0.21µg/ml	24.84µg/ml
Limit of Quantification	1.96 µg/ml	0.48 µg/ml	26.72µg/ml
Resolution	11.69	9.934		
Selectivity	3.65	1.72		

System suitability report

Ruggedness and Robustness:

The ruggedness of the method was determined by carrying out the experiment on different instruments like Shimadzu HPLC (LC 10 AT), Water Alliance 2695 by different operators using different columns. Robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP- HPLC method developed is rugged and robust.

Solution Stability:

In order to demonstrate the stability of both standard and sample solutions during analysis, both solutions were analyzed over a period of 5 h at room temperature. The results show that for both solutions, the retention time and peak area of Ramipril, Atorvastatin and Clopidogrel remained almost unchanged (percentage RSD less than 2.0) and no significant degradation within the indicated period, thus indicated that both solutions were stable for atleast 5 h, which was sufficient to complete the whole analytical process.

System suitability studies:

The column efficiency, resolution and peak asymmetry were calculated for the standard solutions (Table No.5) The values obtained, demonstrated the suitability of the system for the analysis of this drug combinations, system suitability parameters may fall with in ± 3 standard deviation range during routine performance of the method.

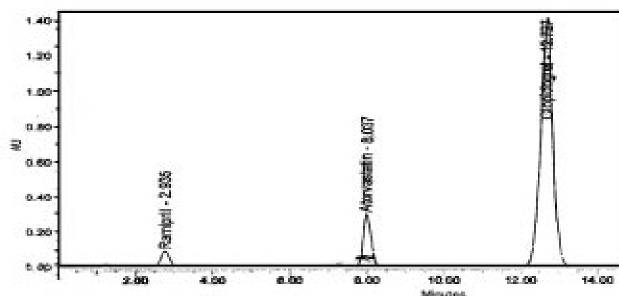

Fig.1: A typical chromatogram for Standards

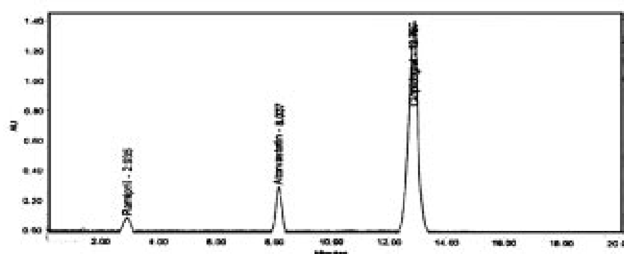

Fig.2: A typical chromatogram standards

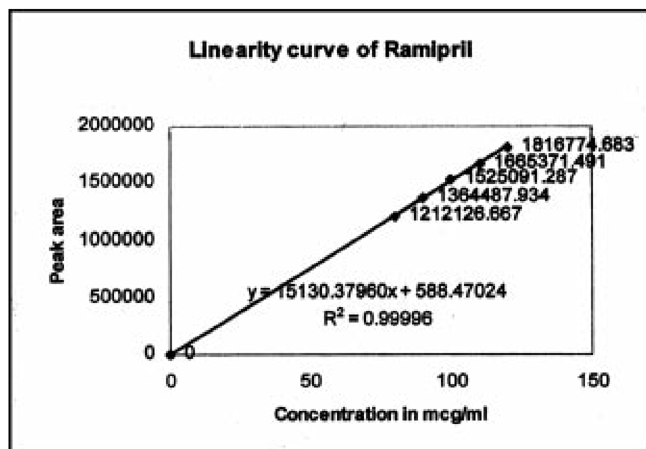

Fig.3: Linearity curve of Ramipril

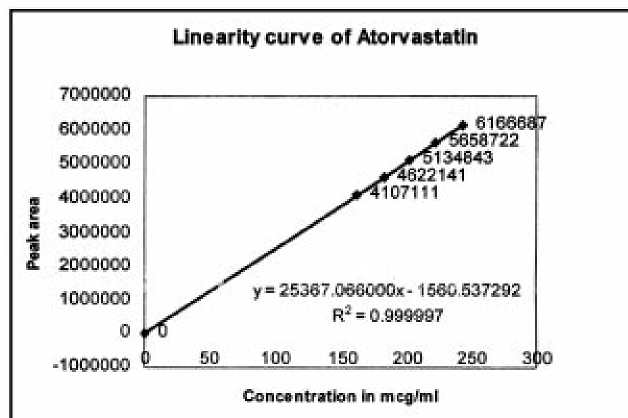

Fig.4: Linearity Curve of Atorvastation

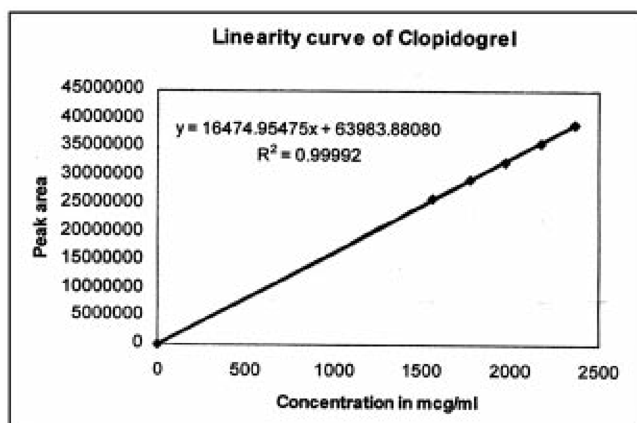

Fig.5: Linearity Curve of Clopidogrel

Conclusion

The proposed RP-HPLC method for the simultaneous estimation of Ramipril, Atorvastatin and Clopidogrel in combined dosage forms is accurate, precise, linear, rugged, robust, simple and rapid. Hence the present RP-HPLC method is suitable for the quality control of the raw materials, formulations and dissolution studies.

References

[1] The Merck Index, 13th edition, Merck and Co., Inc. U.S.A, 2002, PP-8103.

[2] The Merck Index, 12th edition, Merck and Co., Inc. U.S.A, 2002, PP-897.

[3] The Merck Index, 14th edition, Merck and Co., Inc. U.S.A, 2002, PP-2396.

[4] Jemal M, Ouyang Z, Chen BC , Teitz D. Rapid Commun Mass Spectom 1999;11:1003-15.

[5] Bullen WW, Miller RA, Hayes RN. J Am Soc Mass Spectrom 1999;1:55-66.

[6] Lillsunde P. J Chromatogr B Analyt Technol Biomed Life Sci 2004; 806 (2):205-219.

[7] ICH, Q2A Text on Validation of Analytical Procedures, International Conference on Harmonization, Oct. 1994.

[8] ICH, Q3B Validation of Analytical Procedures, Methodology, International Conference on Harmonization, Nov. 1996

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