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Contents

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VIEWS

Prevention of carbon tetrachloride induced hepatotoxicity in rats by alcohol extract of *Capparis zeylanica* stem

SATYANARAYANA. T, ANJANA A MATHEWS*, CHINNA ESWARAIAH. M
College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, India

ABSTRACT

The hepatoprotective activity of the alcoholic extract of *Capparis zeylanica* Linn. (Capparidaceae) stem against carbon tetrachloride induced toxicity was studied in Wistar albino rats. The rats were given a daily pre-treatment with extract (100mg/kg) and silymarin (25mg/kg) orally for 7 days. Hepatotoxicty was induced in rats by administering CCl_4 at dose of 1.25 ml/kg orally on 7th day. The hepatoprotective activity of the extract was confirmed as it produced significant lowering of the elevated serum glutamic oxaloacetic transaminase(SGOT),serum glutamic pyruvate transaminase(SGPT),alkaline phosphatase(ALP) and total bilirubin(TB) and Total Protein when compared with toxic control.

Key Words: Hepatoprotective activity, *Capparis zeylanica* stem, carbon tetrachloride, silymarin.

Introduction

Capparis zeylanica Linn, synonyms *Capparis horrida* Linn., *Capparis brevispina* DC., (Capparaceae),is known as Vyakhranakhi in ayurveda and used in the treatment of several diseases**.** The root bark removes *'Kapha'*, fruits are used to remove '*Tridosha*', bitter removes '*Kapha*' and '*Vata*'. The root bark is used for cooling, as cholagogue and bitter. It is used traditionally as stomachic, sedative, antihydrotic, in cholera, neuralgia, hemiplegia and rheumatism. The seeds and fruits are used in urinary purulent discharges and dysentery. In Northern India, the leaves are used as rubefacient, counterirritant and as cataplasm in boils, swellings and piles [1, 2, 3].

The roots of *C.zeylanica* were reported to have antibacterial, antioxidant activities; also found to act as endothelin receptor antagonists. Significant anti inflammatory and analgesic activity was exhibited by the successive petroleum ether, methanol and aqueous root extracts [4]. The seeds and fruits are reported to have anthelmintic activity. The crude extract of plant was reported to have CNS depressant activity. The steam volatile fraction of flowers and seeds were highly antimicrobial. The 50% alcoholic extract of aerial parts were reported as spasmolytic [2]. The leaves of *C.zeylanica* were found to exhibit immunostimulant activity. The extracts also prevented myelosuppression in mice treated with cyclophosphamide drug [5]. The ethanol and water extracts of leaves showed dose-dependent and significant analgesic activity in several models. The water extract significantly ($P<0.01$) reversed yeast-induced fever [6].

Many species belonging to the genus Capparis were used traditionally in hepatic ailments [2,7-10]. Hence this study was taken up to screen the stem of *C. zeylanica* for hepatoprotective activity against CCl_4 induced hepatotoxicity in Wistar albino rats, in order to ascertain whether this activity could be related to the genus.

Materials and Methods

Plant material

The stems of *C. zeylanica* were collected from Kondappally,Vijayawada district, Andhra Pradesh in the month of March 2006 and were identified and authenticated by Dr. M. Venkaiah, Associate Professor, Department of Botany, Andhra University, Visakhapatnam**.** A voucher specimen was deposited in the Phytochemistry and Pharmacognosy Division, College of Pharmaceutical Sciences, Andhra University.

Chemicals

All chemicals and reagents used were of analytical grade and obtained from Merck Chemical Company, Mumbai, India. The Olive oil was from Figaro Co., Madrid, Spain. The kits for the estimation of SGOT, SGPT, Alkaline phosphatase, Total Bilirubin and Total Protein were purchased from Ranbaxy Fine Chemicals Limited, Diagnostics Division, Himachal Pradesh, India. The standard drug silymarin was a gift sample from Micro Labs, Bangalore, India.

**Address for correspondence:*
mathewanjana@gmail.com

Preparation of the extract and phytochemical screening

Shade dried plant material was packed in a Soxhlet apparatus and extracted exhaustively with 90% alcohol. The extract was concentrated and dried using Rotary Flash Evaporator to give a brown semi solid residue. The alcoholic extract of *C.zeylanica was* screened for the type of phytoconstituents present by various chemical tests for the different classes of compounds.

Animal Treatment

Wistar albino rats (150-200g) obtained from Mahaveera Enterprises, Hyderabad, were maintained on 12h light/dark cycle and allowed food and water *ad libitum*. Animals were randomly divided into four groups of six animals each. Group I served as vehicle control and received 4% acacia suspension. Group II served as toxic control and received 4% acacia suspension. Group III served as standard group and received silymarin (25mg/kg). Group IV served as test group and received alcoholic extract of *C.zeylanica* stem (100mg/kg). All treatments were given orally for 7 days successively. On 7th day, liver toxicity was induced in rats of all groups except group I, with 1:1(v/v) mixture of CCl_4 in olive oil at a dose of 1.25ml/kg, p.o., six hours after last dose [11].

Biochemical Analysis

24hrs after CCl_4 treatment (Day 8) blood was collected from the retro orbital plexus of all the rats. Serum was separated by centrifugation at 2500rpm at 37^0 C for 15min and analyzed for SGPT [12], SGOT [13], ALP [14], TB [15] and Total Protein [16].

Statistical analysis

All the results were expressed as mean ± SEM. One-way Analysis of Variance (ANOVA) was used for the statistical analysis of data. Dunnett's multiple comparison test was used for determining the significance. A probability value of $p<0.05$ was considered as significant. The percentage reduction was calculated by considering the difference between mean values of toxicant and control as 100% reduction.

Results

In the present study, preliminary phytochemical screening of *C.zeylanica* confirmed the presence of sterols, triterpenoids, alkaloids, saponins, flavanoids and glycosides in the alcoholic extract of the stem. The LD_{50} of alcoholic extract of *C.zeylanica* being reported as 1500mg/kg p.o. in mice [17], the test dose selected for the study was 100mg/kg b.w.(1/15 of LD_{50}).

The administration of CCl_4 resulted in significant rise ($p< 0.01$) in SGOT, SGPT, ALP and total bilirubin levels along with significant decrease in total protein ($p<0.01$) levels when compared with Group I (vehicle control) (Table I), which indicates necrosis of liver. The oral administration of alcoholic extract of *C.zeylanica* and silymarin reduced the CCl_4 induced increase in the SGOT, SGPT and TB levels ($p<0.01$) by 72.4% and 85.7%, 68.2% and 73.4%, 95.9% and 97.4% respectively; reversed the depletion of total protein significantly ($p<0.05$,$p<0.01$ respectively) by 35.65% and 41.8% respectively but no significant decrease was observed in ALP levels when compared with GroupII (CCl_4 treated group) (Table I).

In the histopathological studies, the liver sections of rats treated with vehicle showed normal hepatic architecture (fig1),whereas that of CCl_4 treated group showed total loss of hepatic architecture with intense peripheral central vein necrosis, fatty changes, congestion of sinusoids, kupffer cell hyperplasia, crowding of the central vein and apoptosis (fig2). In the case of rats treated with silymarin (fig3) and those pre-treated with *C.zeylanica* alcoholic extract (fig4), the liver showed normal hepatic architecture with only moderate accumulation of fatty lobules and mild degree of cell necrosis, clearly indicating the protection offered by standard drug silymarin and the plant extract.

Table- 1

Effect of alcoholic extract of *C.zeylanica* stem on carbon tetrachloride induced hepatotoxicity in rats

Groups	SGOT(U/L) (% Reduction)	SGPT(U/L) (% Reduction)	ALP(U/L) (% Reduction)	TB(mg/dl) (% Reduction)	Total Protein(g/dl) (% Reduction)
I Vehicle Control	81.77±2.48	40.0±1.93	190.4±6.46	0.052±0.01	10.88±0.23
II CCl_4 Control	353.7±2.48^a	261.5±5.50^a	257.7±4.65^a	0.285±0.03^a	9.18±0.09^a
III Silymarin + CCl_4	120.7±6.13^b (85.7 %)	99.02±7.71^b (73.4 %)	250±6.21	0.058±0.01^b (97.4 %)	9.89±0.16^b (41.8 %)
IV *C.zeylanica* ext + CCl_4	156.8±5.94^b (72.4%)	110.5±4.29^b (68.2 %)	239 ± 6.96	0.062±0.06^b (95.9 %)	9.79±0.08^c (35.65 %)

Values are mean ± SEM, N= 6, ^{a} $p \leq 0.01$ compared with Group I. ^{b} $p \leq 0.01$, ^{c} $p \leq 0.05$ compared with Group II.

Photomicrographs representing effect of test materials against carbon tetrachloride induced hepatotoxicity in rats.

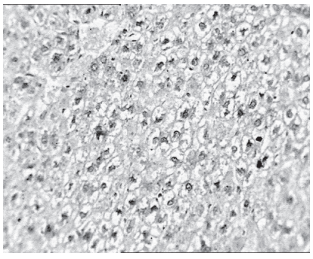

Fig. 1-Vehicle control

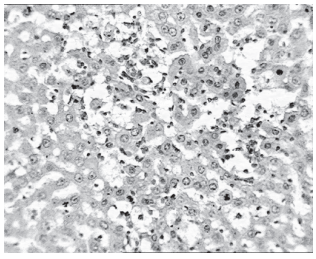

Fig. 2-Toxic control

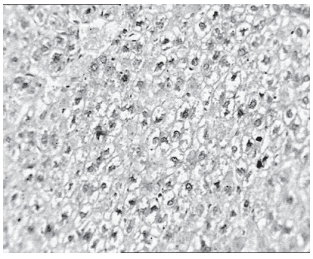

Fig. 3: Silymarin treated

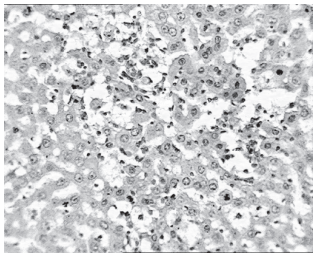

Fig. 4: *C.zeylanica* treated

Discussion

Carbon tetrachloride induced hepatotoxicity is commonly used as an experimental method to study the hepatoprotective activity of plant extracts. Highly reactive trichloro free radical formation, which attacks polyunsaturated fatty acids of the endoplasmic reticulum, is responsible for the hepatotoxicity of CCl_4 [18] It produces hepatotoxicity by altering liver microsomal membranes in experimental animals [19].

From the table it is clear that the plant extract was able to reduce all the elevated biochemical parameters due to CCl_4 intoxication. The reduction in levels of SGOT and SGPT towards normal value is an indication of regeneration process. Reduction of ALP levels with simultaneous depletion of raised bilirubin levels indicates the stability of the biliary function during CCl_4 injury. Reduction in level of total protein due to CCl_4 induced hepatotoxicity is due to the initial damage produced and localized in the endoplasmic reticulum which results in the loss of P_{450} leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides leading to fatty liver [18]. The rise in protein levels suggest the stabilization of endoplasmic reticulum leading to protein synthesis.

Seven days pretreatment with the test extract (100mg/kg p.o.) protected the animals significantly ($p<0.01$) from carbon tetrachloride induced hepatotoxicity as compared to toxic control, and clearly indicates the hepatoprotective activity of the alcoholic extract of *C. zeylanica* stem.

Different components including ²-sitosterol and alkaloids have been isolated from the plant [20, 21]. In the present work, presence of sterols, alkaloids, saponins and glycosides in the alcoholic extract were confirmed. Reports of ²- sitosterol and alkaloids as hepatoprotective agents have been published [22, 23]. Hence the hepatoprotective activity of the *C.zeylanica* alcoholic extract may be due to the presence of these compounds.

The results of the present study suggest that alcoholic extract of *C.zeylanica* stem at dose of 100mg/kg, p.o. showed significant hepatoprotective activity which may have co-relation to plants of this genus.

Acknowledgement

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Venlafaxine desmethylation–oxidative metabolism using microbes

[1]VIDYAVATHI. M*, [1]PRASAD. KVSRG,[2] KRISHNA. DR
[1]School of Pharmaceutical Sciences, Sri Padmavathi Mahila Visva Vidyalayam, Tirupati - 517 502, A.P., India.
[2]Clinical research Division, Covidien Inc. Hazelwood, MO 63146, USA

ABSTRACT

The present work was aimed at developing a microbial model for easy synthesis of metabolite of venlafaxine, which has comparable therapeutic activity to that of parent drug and to study the metabolism of other drugs metabolized by CYP2D6 and CYP3A4. The metabolites of venlafaxine in microbial cultures were identified, isolated and the structures were confirmed using fermentation techniques, HPLC and LCMS methods. Among different organisms screened *Saccharomyces cervisiae* showed extra peak at 8.3 min. compared to its controls in HPLC indicating formation of metabolite by *Saccharomyces cervisiae*. The metabolite was characterized by Liquid Chromatography Mass Spectrometry and was found as glucuronic acid conjugate of tridesmethyl venlafaxine. O-desmethylation of venlafaxine by CYP 2D6 and N-desmethylation of venlafaxine by CYP 3A4 and formation N,O-didesmethyl venlafaxine were reported in human beings. Among the tested organisms *Saccharomyces cervisiae* was able to metabolise venlafaxine by oxidative desmethylation like human CYP2D6 and CYP3A4 and the oxidized metabolite was conjugated by glucuronidation. Hence, this study demonstrates a microbial model to produce metabolite of venlafaxine by oxidative desmethylationion as mediated by human CYP2D6 and CYP3A4.

Keywords : Microbial model, venlafaxine, Oxidative desmethylation, CYP2D6 and CYP3A4, desmethyl venlafaxine

Introduction

Metabolism refers to the structural modification of drugs and chemicals by enzymatic systems, and understanding of drug metabolism plays very important role in the development of new drug entities. Traditionally, drug metabolism studies were conducted on small animal models, perfused organs [1,2] *in vitro* enzyme systems and *in vitro* cell cultures.

Recently microbial models are being introduced in drug metabolism studies as *in vitro* models for the prediction of mammalian drug metabolism with successful applications [3-5]. The use of microorganisms as models of mammalian metabolism has been well documented [6-9]. The studies on steroids and antibiotics led to the formalization of the microbial models of mammalian metabolism by Smith and Rosazza [4]. Many scientists have also published several reviews and updates on this topic [1,2,10,11].

One of the most important drug metabolizing enzyme systems in humans is the cytochrome P450 (CYP) a super family containing more than 1000 enzymes [12], which are responsible for oxidative metabolism of numerous endogenous compounds and xenobiotics. In mammals and microorganisms, enzymatic hydroxylation of xenobiotics is often carried out by cytochrome P-450 [13].

In present study venlafaxine a second generation novel phenethylamine antidepressant is selected [14-16]. It is well absorbed and extensively metabolised in the liver by cytochromeP450 2D6 [17,18] produces O-desmethyl venlafaxine (ODV) which is active comparable to that of parent drug [19-21].

Venlafaxine is also metabolised to two less active metabolites N-desmethyl by CYP 3A4 and N,O-didesmethyl venlafaxine [22]. The major metabolites of venlafaxine found in mouse, rat, dog, rhesus monkey and man are different. N,O-didesmethylvenlafaxine glucuronide in mouse, cis-1,4-dihydroxy venlafaxine in rat, O-desmethyl venlafaxine glucuronide in dog, N,N,O-tridesmethyl venlafaxine in monkey and O-desmethyl venlafaxine in man were found [23]. The mammalian metabolic pathway of venlafaxine is shown in fig. 1.0.

Therefore, the present study was aimed at developing a microbial model to use as tool for producing metabolites venlafaxine that are usually produced by CYP3A4 and 2D6 in human, as microbial systems have more advantages than existing *in vitro* and *in vivo* models.

**Address for correspondence:*
vidyasur@rediffmail.com

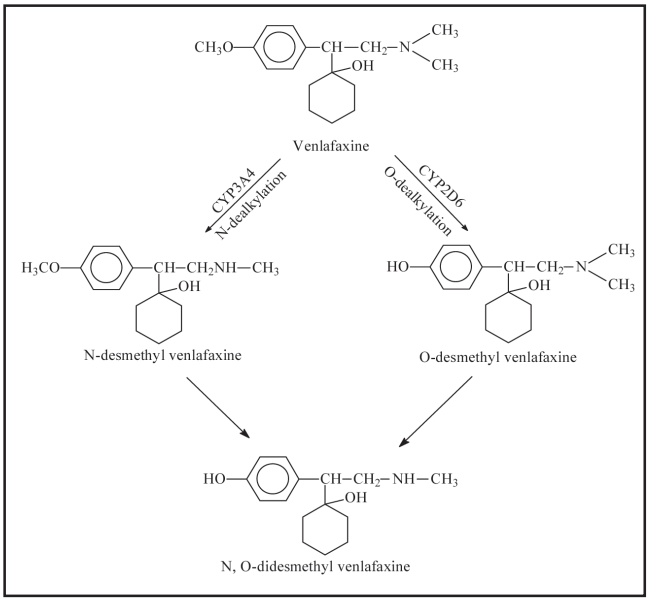

Fig. 1.0 : Mammalian metabolic pathway of venlafaxine

Materials and Methods

Microorganisms

Cultures were obtained from National Chemical Laboratories, Pune, India. The cultures used in the present work were *Proteus vulgaris* (NCIM 2027), *Pseudomonas aeruginosa* (NCIM 2053), *Nocardia hydrocarbonoxydans* (NCIM 2386), *Cunninghamella elegans* (NCIM 689) and *Saccharomyces cerevisiae* (NCIM 3090).

Chemicals

venlafaxine was obtained from M/s. Vimta labs, Hyderabad.. Chloroform, Isopropanol, n-Heptane, Disodium hydrogen phosphate, Ammonium acetate, Acetic acid were purchased from S.D. fine chemicals, Mumbai, India. Acetonitrile was obtained from Spectrochem Pvt. Ltd., Mumbai, India.

Fermentation procedure: The experiments were carried out using respective sterile growth media for different microorganisms consisting of nutrient broth for bacteria, MGYP broth for yeast and potato dextrose broth for fungi. Microbial metabolism studies were carried out by shake flask cultures in a shaker incubator, operating at 120 rpm at 32°C. The experiments were carried out in culture flasks (250 ml) each containing 50 ml. growth medium. Fermentations were carried out according to standard protocol [24]. In brief, the substrate (venlafaxine) 1% (w/v) was added to the culture medium of selected organisms at a concentration of 10 µg/ml of medium in samples and incubated on a shaker. The study also maintained substrate control and culture controls. The incubation was continued for 24h to 48 h for bacteria and fungi respectively.

Extraction procedure: A clear supernatant liquid was collected from each flask and drug and metabolites were extracted with mixture of chloroform, isopropanol and n-heptane in the ratio of 60:14:26. [21]. The dried extracts were reconstituted with 0.5 ml. of mobile phase and centrifuged at 13,000 rpm for 8 minutes using biofuge fresco centrifuge (Heraeus, Germany). 20 µl. of the supernatant of all samples blank I (Drug control), blank II (Culture control) and sample of each culture were spiked into column after spiking control composed of pure drug solution to identify venlafaxine retention time.

Analytical techniques:

Chromatographic procedure : High performance liquid chromatography (HPLC) analysis was carried out using a HPLC system (Shimadzu, Kyoto, Japan) consisted of LC-10A solvent delivery module and SPD-10AVP UV-visible spectrophotometric detector and a Wakosil II5C-18RS-100a. 5UM, 4.6 250 MM SS column (SGE Japan). Sensitivity was set at 0.001 aufs. Mobile phase consisted of acetonitrile and 0.01 M ammonium acetate buffer of pH adjusted with acetic acid to 3.9 at ratio of 25:75% v/v was used at a flow rate of 1 ml/min. Elution was monitored using a UV/Vis detector set at 200 nm [19,21].

Mass spectrometry*:* Mass spectral data were obtained using Liquid Chromatography Mass Spectrometry (Agilent technologies, Germany). Model is LC/MSD - trap SL-1100 series, LC coupled to a mass spectrometer operating in the electron spray ionisation (ESI) mode. Ionisation was carried out in positive ion mode using Ion trap detector (3.5 KV, 325°C, 40 psi) with same mobile phase.

Results

Screening of cultures & identification of drug and metabolite**:** Five microorganisms were screened in the present study. The microbial transformation and control samples were extracted and analyzed as described above. It is found that the chromatograms of the blank culture controls showed no metabolites of venlafaxine. Blank substrate control showed only the presence drug and no metabolite of venlafaxine from Table1. As shown in Fig. (**2**), a metabolite was detected in *Saccharomyces cerevisiae* culture, compared with the control cultures. *Saccharomyces cerevisiae* supported biotransfomation of venlafaxine. (Fig. (**2**). Since, *Saccharomyces cerevisiae* biotransformed the venlafaxine, it was used for further investigation..

Following the transformation of venlafaxine by *Saccharomyces cerevisiae*, metabolite was isolated by elute from HPLC, and its structure was identified by LC/MS analysis.

Parent drug**:** The compound eluting at 10.6min. was identified as venlafaxine by comparison of the retention time with pure drug (Table 1). Venlafaxine could generate a molecular ion $(M+H)^+$ at m/z 278 which was supported by fragment ion peak at m/z 260,obtained by loss of water molecule in its mass spectrum Fig.(**3**)

Metabolite**:** Metabolite was eluted at 8.3min. in HPLC which

Table-1

HPLC data for venlafaxine and its metabolite from microbial culture extracts

Organism	Retention time in minutes			
	(Blank I) Drug control	(Blank II) Culture control	(Control) Pure venlafaxine	Sample
Proteus vulgaris	2.1 3.7 5.3 10.6	2.1 3.7 5.3 -	2.1 - - 10.6	2.1 3.7 5.3 10.6
Pseudomonas aeruginosa	2.1 3.7 5.3 10.6	2.1 3.7 5.4 -	2.1 - - 10.6	2.1 3.7 5.3 10.6
Nocardia hydrocarbonoxydans	2.1 3.7 5.4 10.6	2.1 3.7 5.4 -	2.1 - - 10.6	2.1 3.7 5.4 10.6
Cunninghamella elegans	2.8 10.6	2.8 -	2.1 10.6	2.8 10.6
Saccharomyces cerevisiae	3.1 3.7 - 10.6	2.1 3.7 - -	2.1 - - 10.6	2.1 3.7 8.3* 10.6

* - Metabolite peak

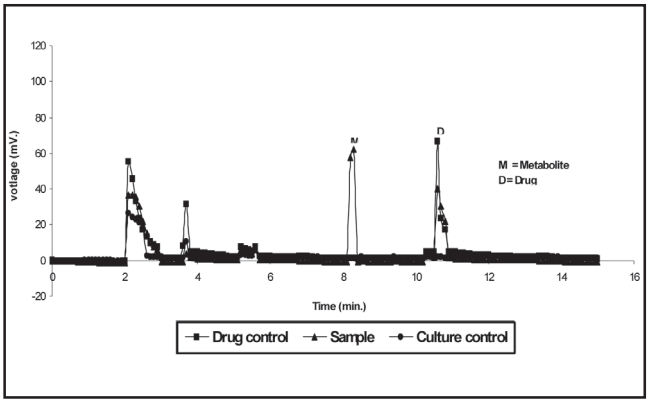

Fig. 2: HPLC chromatogram of venlafaxine from culture extracts of Saccharomyces cerevisiae

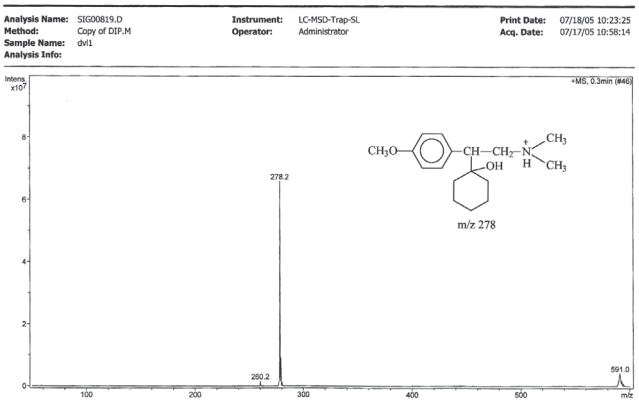

Fig. 3: Mass spectrum of venlafaxine

was found only with sample of *Saccharomyces cerevisiae.* Metabolite was isolated and analysed by LCMS, it has the molecular ion at m/z 413 $(M+H)^+$ in its mass spectrum with the support of fragment ion peaks at m/z 391,364,321,204,129,107 as shown in Fig**. (4).** its mass fragmentation pattern is shown in Fig. **(5).**

Microbial transformation of venlafaxine and comparison with mammalian metabolism: In the present study, venlafaxine was transformed by *Saccharomyces cerevisiae* to the metabolite glucuronide conjugate of tridesmethyl venlafaxine. The structure of metabolite and proposed metabolic pathway in *Saccharomyces cerevisiae* is shown in Fig**. (6),** and is compared with metabolic pathway identified in human (fig.1).

Discussion

In this study, five microorganisms were screened for their potential to metabolize venlafaxine, a probe for CYP 2D6 and CYP 3A4 activity [17,22]. *Saccharomyces*

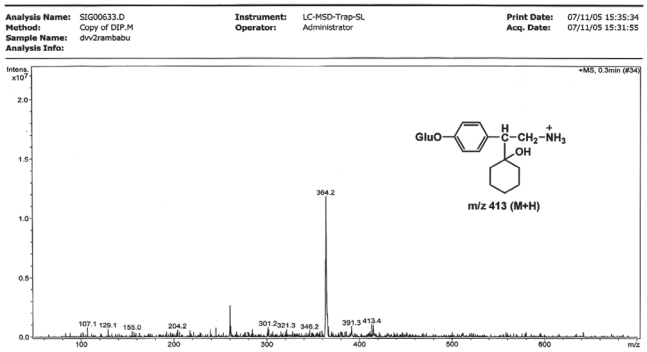

Fig. 4: Mass spectrum of venlafaxine metabolite formed by *saccharomyces cerevisiae*

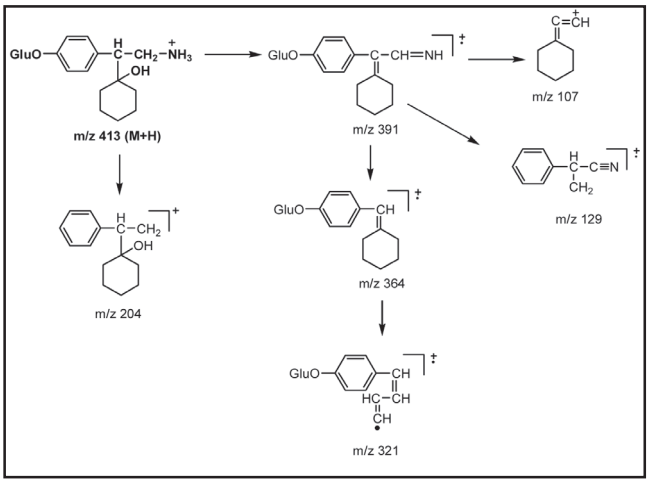

Fig. 5: Mass fragmentation pattern of venlafaxine metabolite produced by *Sacccharomyces cerevisiae*

cerevisiae showed an ability to convert venlafaxine into desmethylvenlafaxine. It was reported that, venlafaxine metabolised to one active metabolite O-desmethyl venlafaxine by CYP 2D6 and two less active metabolites N-desmethyl by CYP 3A4 and N,O-didesmethyl venlafaxine in human beings [17,18,22]. In the present study glucuronidation of O- desmethyl venlafaxine was observed which was supported by fragment ion peaks at m/z 391 and 364.

Different metabolites of venlafaxine have been reported in mammals like mouse, rat, dog, monkey and man involving dealkylation, glucuronide conjugation as major metabolic reactions. Glucuronide conjugate of desmethyl venlafaxine was found in mouse and dog [24].

Based on the above results, from the proposed metabolic pathway of venlafaxine by *Saccharomyces cerevisiae* (fig. 6), it was observed that the *Saccharomyces cerevisiae* metabolised venlafaxine by dealkylation as reported in man and animals and glucuronide conjugation as found in mouse and dog.

In conclusion the desmethylvenlafaxine an active human metabolite of venlafaxine was formed by *Saccharomyces*

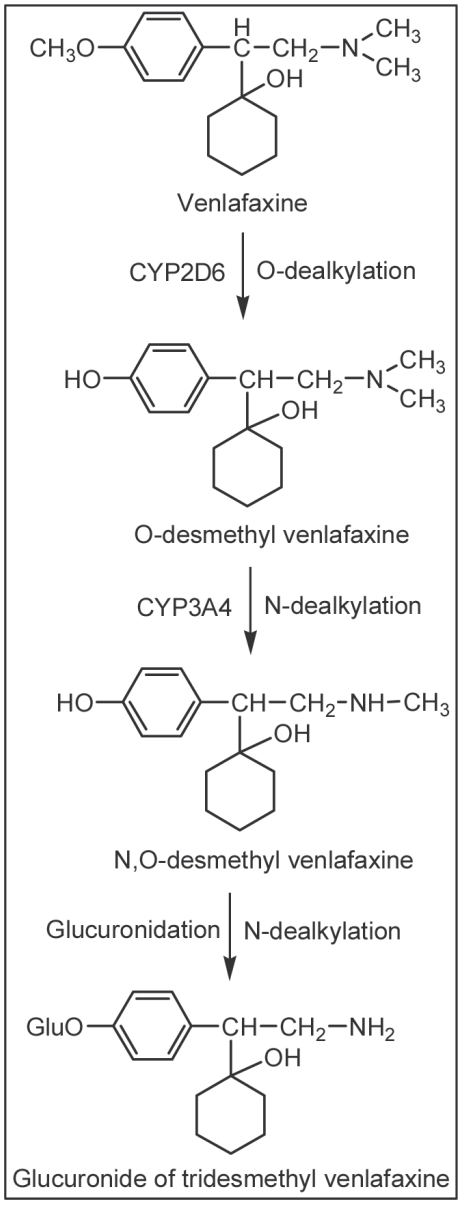

Fig.6: Proposed metabolic pathway of venlafaxine by *Saccharomyces cerevisiae*

cerevisiae. The ability of *Saccharomyces cerevisiae* to mimic the mammalian metabolism and to produce active metabolite in large quantities [25] indicates that microbial systems represent an attractive alternative to the use of mammalian systems in the preclinical DMPK studies and for synthesis of metabolite in large scale [26].

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Synthesis and Antimicrobial Screening of certain Novel Bases Bearing Pyrazolone Moiety

KISHORE KUMAR. K*, CHANDRASHEKAR. K.B*, JAYAVEERA. K.N*, SAIDUREDDY. S**, NARAYANARAO. D.V**, SPOORTHY. Y.N**, RAVINDRANATH. L.K**

* Department of Chemistry J.N.T.U., O.T.R.I, Anantapur 515 002, Andhra Pradesh, India

** Department of Chemistry S.K.University, Anantapur, Andhra Pradesh, India.

ABSTRACT

Treatment of [3-trifloromethyl-5-oxo-4-(4'-substituted aryl hydrazono)-4,5-dihydro-pyrazol-1-yl]-acetic acid hydrazide **(4)** with Isatin affords, [3-trifloroMethyl-5-oxo-4-(4'-substituted aryl hydrazono)-4,5-dihydro-pyrazol-1-yl]-acetic acid (2-oxo-1,2-dihydro–indol-3-ylidene)–hydrazide **(5)**, Amino Methylation of (5) with formaldehyde and cyclic secondary amines furnishes [3-trifloroMethyl-5-oxo-4-(4'-substituted aryl hydrazono)-4,5-dihydro-pyrazol-1-yl]-acetic acid (2-oxo-1-piperidine/ morpholine/N–methylpiperazine-1-ylmethyl-1,2-dihydro–indol-3-ylidene)- hydrazide **(6)**.

Key Words: Mannich bases, Aryl hydrazano pyrazolo isatin derivatives, Antimicrobial activity.

Introduction

Pyrazole and pyrazoline derivatives are a class of biologically active compounds which have been associated with anti–microbial activity [1–5], hypo–glycemic activity [6] anti–inflammatory activity [7,8], Insecticides, pesticides [6–11] and dyestuffs [12]. Isatin derivatives have gained unique importance due to broad spectrum of pharmacological activities which are reflected by their use as antibacterial [13], antihelmintic [14] anticonvulsants [15] and CNS depresent agents [16]. The antibacterial activity [17–18] of Mannich bases has been well established, in view of these observations and in continuation of our work on the synthesis of biological active pyrazolones, it appeared of interest to synthesize some novel Mannich bases bearing pyrazolone and indole moieties. The structure of the compounds(4–6) have been established on the basis of their elemental analysis and spectral data. (I.R, H^1–NMR, and MS).

Experiment

M.P. were recorded on a cintex m.p. apparatus, in open capillaries and are uncorrected. I.R spectra were recorded in KBr on Perkin–Elmer BX series FT–IR Spectrometer, H^1–NMR spectra on a Varian Germany – 200MHz Spectrometer using TMS as internal standard (Chemical Shift in –δppm) and Mass Spectrometer. Purity of the compounds was checked by TLC.

**Address for correspondence*
vickishores@gmail.com

Preparation of [3-trifloromethyl-5-oxo-4-(4'-substituted aryl hydrazono)-4,5-dihydro-pyrazol-1-yl]-acetic acid hydrazide (4a–f)

A solution of sodium acetate (1 gm) in 100 ml of Aqueous alcohol (50%) is added to a solution of trifloro ethyl acetoacetate (0.1 mole) in 500 ml of ethanol at 0^0C. To this cold mixture, the corresponding diazonium chloride is added gradually till turbidity is observed. The addition is continued till yellow crystals of phenyl diazonium trifloro acetoacetate is separated out (1a–f). The crystals are filtered, washed with water and dried.

Preparation of 2a-f

Condensation of 4–substituted arylhydrazano trifloro acetoacetate (1a), hydrazine and dimethyl formamide (10 drops), Al_2O_3 was subjected to microwave irradiation at 150W intermittently at 30sec intervals for 2 minutes. After complete conversion as indicated by the TLC, the reaction mixture was cooled and heated with water. The precipitate 3–trifloromethyl-4-(4'-substituted aryl hydrazano)-pyrazoline-5-one (2a–f) was filtered, recrystallized from ethanol to give 2a, m.p 187^0C, yield 88%.

Preparation of 3a-f

To a solution of (0.01 mole) of 2a, anhydrous K_2CO_3, DMF was stirred at room temperature for 8 hours. The reaction mixture was diluted with ice cold water. The separted solid was identified as [3–trifloromethyl-5-oxo-4-(4'-substituted aryl hydrazono)-4,5-dihydro-pyrazol-1-yl] acetic acid ethyl ester (3a). A solution of 0.01 mole of 3a and hydrazine hydrate in ethanol was refluxed for 5 hrs.

The reaction mixture was cooled and poured on to ice cold water with stirring. The separated solid [3-trifolormethyl-5-oxo-4-(4'-phenyl hydrazono)-4,5-dihydro-pyrazol-1-yl]-acetic cid hydrazide (4a) was filtered washed with water and recrystallized from ethanol to afford 4a, m.p.-160^{0}C, yield 70%.

IR (KBr) : 3445 & 3425 (NH_2), 3305 (NH), 1620 (C=N), 1665 (C=O). H^1-NMR ($CDCl_3$ + CH_3OH-d_6) : 2.3 (S, 2H, NH_2), 8.6 (S, 1H, NH), 7.2 (S, 1H, Ar-NH), 7.5–7.6 (m, 5H, C_6H_5), 4.05 (S, 2H, N–CH_2–CO). MS: M/Z 328 (M^+,37.3%), 298 (31.3%) 270 (9.8%), 258 (6.4%), 256 (4.7%), 237 (10.5%), 224 (100%), 91 (39%), 72 (27.4%),

Other compounds 4b–f were synthesized similarly.

Preparation of [3-trifloro methyl-5-oxo-4-(4'-substituted aryl hydrazono)-4,5-dihydro-pyrazol-1-yl]-acetic acid (2-oxo-1,2-dihydro–indol-3-ylidene)–hydrazide (5a–f)

The synthon, isatin was prepared by the procedure described by Marvel and Heins [19]. A mixture of 4a (0.01 mole) and isatin (0.01 mole) heated in DMF solvent on water bath for 45 minutes. The DMF was distilled OH and residue was poured onto crushed ice. The solid thus obtained is washed with water and recrystallized from aqueous methanol to furnish **(5a)**.

IR (KBr) : 3205 (NH), 3170 (Indole NH), 1602 (C=N), 1656 (C=O), 1700 (Indole C=O), 1618 (CO–NH); ^{1}HNMR ($CDCl_3$) : 7.4 (S, 1H, Ar–NH), 10.11 (S, 1H, CONH), 5.98 (S, 2H, N–CH_2–CO), 12.95 (S, 1H, Indole NH), 7.4–7.92 (m, 9H, Ar–H). MS: m/z 457 (M^+,12.7%), 366(32.3%) 351 (100%) 312 (66.7%), 298 (31.3%) 270 (9.8%), 256 (4.7%), 201 (39.7%), 160 (17.5%), 146 (9.8%), 106 (14.4%), 91 (39%)

Other compounds 5b–f were synthesized similarly and their characterization data are given in Table Ia, Ib.

Preparation of [3-trifloromethyl-5-oxo-4-(4'-substituted aryl hydrazono)-4,5-dihydro-pyrazol-1-yl]-acetic acid (2-oxo-1-piperidine/morpholine/N–methylpiperazine-1-ylmethyl-1,2-dihydro–indol-3-ylidene)- hydrazide (6a–c)

A mixture of 5a is stirred with aqueous formaldehyde piperdine in DMF for 6 hrs at room temperature and diluted with water. The solid thus separated was filtered, washed with water and recrystallized from ethanol to give 6a, m.p. 163^{0}c, yield, 70%.

IR(KBr) : 3195 (NH), 1610 (C=N), 1676 (C=O), 1720 (Indole C=O), 1654 (CO–NH), 2933 (CH_2); ^{1}HNMR (200MHz, $CDCl_3$ + DMSO–d_6), 1.45 (m, 6H(CH_2)$_3$), 2.56 (t, 4H, –CH_2–N–CH_2), 4.45 (S, 2H, –N–CH_2–N–), 9.5 (S, –CONH), 7.15(S, 1H, Ar–NH), 5.9 (S, 2H, N–CH_2–CO), 7.25–7.8 (m, 9H, Ar–H).

MS: m/z 554 (M^+, 36.5%), 469 (13.5%), 463 (36.3%), 457 (24.5%), 448 (19.4%) 393 (100%), 312 (66.7%), 298 (32.3%), 256 (31.3%) 242 (44.3%), 222 (26.7%), 178 (14.5%), 106 (14.4%) 97 (13.4%), 85 (10.4%).

Other members of the series 6b and 6c were similarly prepared and their characterization data are given in Table II a, & b.

Biological activity:

Anti Bacterial Activity

Compounds 6a, 6b and 6c were evaluated for their antibacterial activity *invitro* against *Staphylococcus aureus*, *Bacillus cereus*, *Escherchia coli*, *Pseudomonas aeruginosa*

Table Ia

^{1}HNMR Spectral date of – 5a -f

Compound	R	^{1}HNMR (200MHz) ($CDCl_3$+DMSO-d_6) (δppm)
5b	CH_3	0.95 (s, 3H, CH_3), 6.96 (s, H, Ar - NH), 10.95 (s, 1H, CONH), 5.82 (s, 2H, N-CH_2 – CO), 12.75 (s, 1H, Indole NH) 6.73 – 7.2 (m, 4H, Ar – H), 7.58 (d, 2H) and 7.65 (d, 2H)
5c	OCH_3	3.24 (s, 3H, OCH_3), 6.92 (s, 1H, Ar - NH), 10.95 (s, 1H, CONH), 5.8 (s, 2H, N-CH_2CO), 12.75 (s, 1H, Indole NH) 6.73 – 7.2 (m, 4H, Ar – H), 7.5 (d, 2H) and 7.6 (d, 2H)
5d	OC_2H_5	1.15 (t, 3H, CH_3), 3.16 (q, 2H, O-CH_2), 6.98 (s, 1H, Ar – NH), 10.91 (s, H, CO – NH), 5.76 (s, 2H, N-CH_2CO) 12.72 (s, 1H, Indole NH)6.73 – 7.2 (m, 4H, Ar – H), 7.60 (d, 2H) and 7.70 (d, 2H)
5e	Cl	6.98 (s, H, Ar – NH), 10.93 (s, H, CO – NH), 5.8 (s, 2H, N-CH_2CO), 12.7 (s, 1H, Indole NH), 6.73 – 7.2 (m, 4H, Ar – H), 7.65 (d, 2H) and 7.76 (d, 2H)
5f	Br	6.98 (s, H, Ar – NH), 10.93 (s, H, CO – NH), 5.78 (s, 2H, N-CH_2CO) 12.75 (s, H, Indole NH), 6.73 – 7.2 (m, 4H, Ar – H), 7.53 (d, 2H) and 7.6 (d, 2H)

Table IIb
Characterization data of 5(a-f)

Compd	R	M.P.°C	Yield (%)	Mol. formula	Found (%) (Calcd)				
					C	H	N	Cl	Br
5a	H	219	75	$C_{20}H_{14}N_7O_3F_3$	60.15 (60.00)	3.65 (3.50)	24.45 (24.50)	–	–
5b	CH_3	245	74	$C_{21}H_{16}N_7O_3F_3$	60.99 (60.86)	3.96 (3.86)	23.80 (23.67)	–	–
5c	OCH_3	238	75	$C_{21}H_{16}N_7O_4F_3$	58.90 (58.60)	3.80 (3.72)	22.95 (22.79)	–	–
5d	OC_2H_5	230	80	$C_{22}H_{18}N_7O_4F_3$	59.62 (59.45)	4.20 (4.05)	22.21 (22.07)	–	–
5e	Cl	228	82	$C_{20}H_{13}ClN_7O_3F_3$	55.43 (55.93)	3.15 (2.99)	22.78 (22.55)	8.25 (8.17)	–
5f	Br	248	80	$C_{20}H_{13}BrN_7O_3F_3$	50.10 (50.11)	2.80 (2.71)	20.64 (20.46)	–	16.75 (16.68)

Table IIa
^{1}HNMR Spectral data of – 6(b –c)

Compd	R	X	^{1}HNMR (200MHz, $CDCl_3$+DMSO-d_6) (δppm)
6b	H	O	2.62 (t, 4H, CH_2-N-CH_2), 3.70 (t, 4H, CH_2-O-CH_2), 4.50 (s, 2H, N-CH_2-N-), 6.00 (s, 2H, N-CH_2-CO-), 9.5 (s, 1H, CONH), 7.15 (s, 1H, Ar - NH), 6.8 – 7.62 (m, 9H, Ar – H)
6c	H	N-CH_3	2.42 (t, 4H, CH_2-N-CH_2), 2.70 (t, 4H, CH_2-N-CH_2), 4.20 (s, 3H, N-CH_3), 4.52 (s, 2H, N-CH_2-N-), 5.99 (s, 2H, N-CH_2-CO-), 9.93 (s, 1H, CONH), 7.15 (s, 1H, Ar – NH), 6.95 – 7.6 (m, 9H, Ar – H)

Table II b
Characterization data of 6 (a-c)

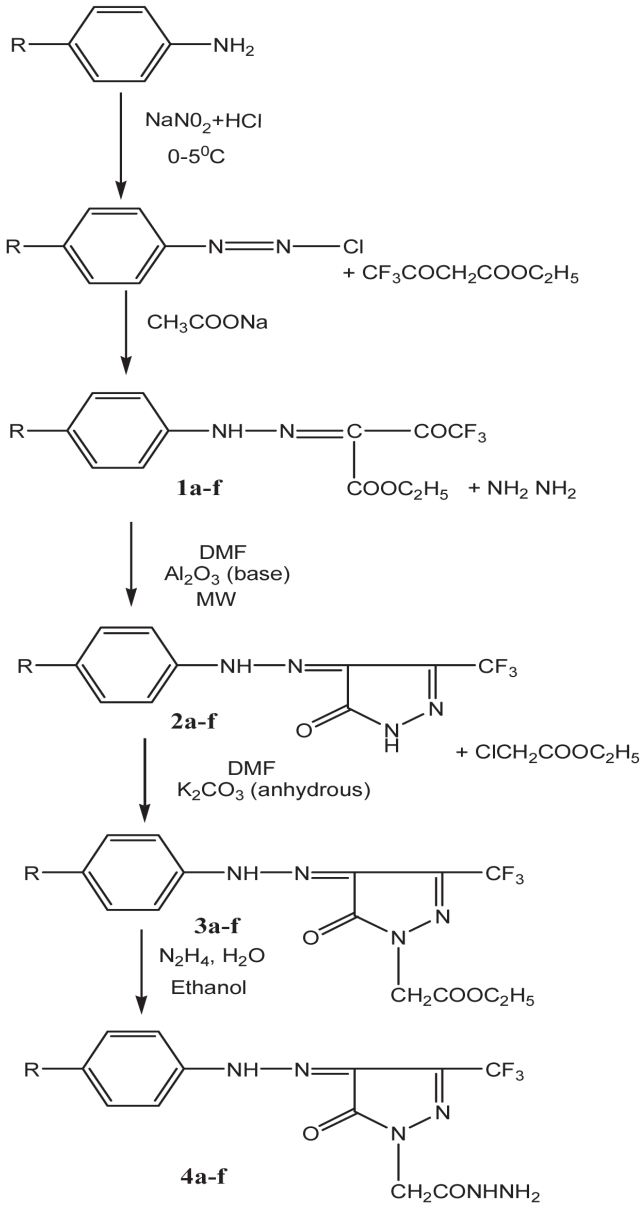
Compd	R	X	M.P.	Yield (%)	Mol. formula	Found (%) calculated				
						C	H	N	Cl	Br
6a	H	CH_2	163	75	$C_{26}H_{25}N_8O_3F_3$	62.99 (62.77)	5.15 (5.00)	22.70 (22.53)	–	–
6b	H	O	165	88	$C_{25}H_{23}N_8O_4F_3$	60.25 (60.12)	4.50 (4.60)	22.65 (22.44)	–	–
6c	H	NCH_3	162	85	$C_{26}H_{26}N_9O_3F_3$	60.72 (60.98)	5.27 (5.07)	24.85 (24.60)	–	–

using the filter paper disc method [20] at 500 µg/disc concentration. The activity was compared with known standard amoxcyllin. All the compounds showed varied degree of antibacterial activity. All the compounds were for less active than the standard drug taken.

Anti Fungal Activity

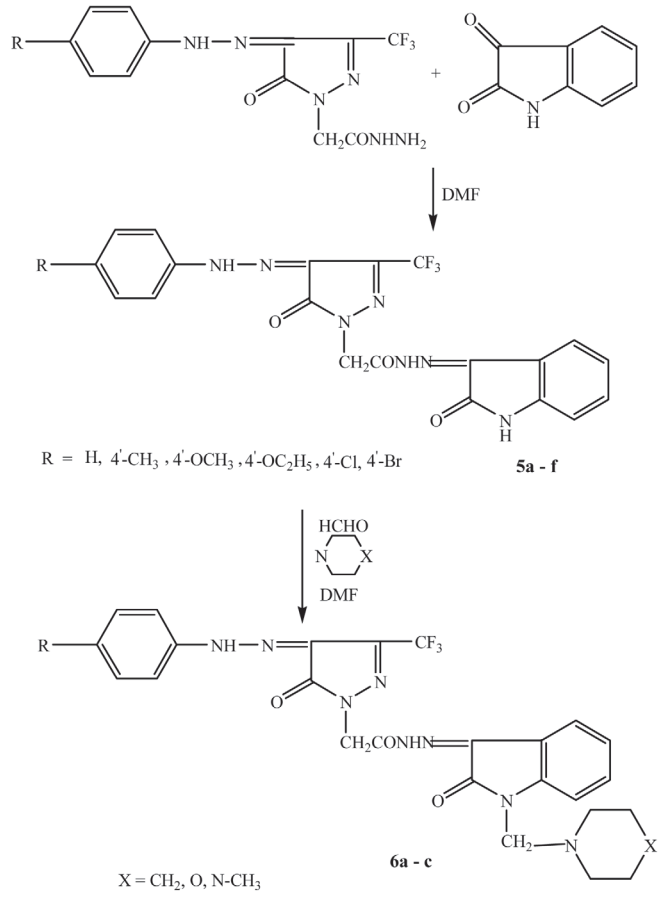
Compounds 6a, 6b and 6c were evaluated for their antifungal activity *invitro* against *Aspergillus niger*, *Candida albicans* using the filter paper disc method at 500 µgm/disc concentrations. The activity was compared with known standard ketoconazole. All the compounds were far less active than the standard drug taken.

Scheme – I



R = H, 4'–CH_3, 4'–OCH_3, 4'–OC_2H_5, 4'–Cl, 4'–Br

Scheme – I (continuation)



R = H, 4'-CH_3 , 4'-OCH_3 , 4'-OC_2H_5 , 4'-Cl, 4'-Br

X = CH_2, O, N-CH_3

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Direct and first order derivative spectrophotometric determination of Cobalt (II) using Bis Vanillin thiocarbohydrazone(BVTH)

RAMAKRISHNA REDDY. S, DEVANNA. N*, JAYAVEERA. K.N, AND CHANDRASEKHAR. K.B
Department of Chemistry, Jawaharlal Nehru Technological University, Anantapur,
Anantapur-515002, Andhra Pradesh, India.

ABSTRACT

Bis Vanillin thiocarbohydrazone (BVTH) used as chromogenic reagent for spectrophotometric determination of Cobalt (II).BVTH reagent gives bright yellow coloured water-soluble complex in acidic buffer medium. The maximum absorbance is observed in the pH range 6.0-7.0. The molar absorptivity and Sandell's sensitivity of Co(II) at λ_{max} 394 nm was found to be 3.2 x 10^4 L. mol^{-1} cm^{-1} and 0.0018 μg/cm^2. Beer's law validity range is from 0.0589 to 1.1786μg/ml. Cobalt forms 1:2 complex with BVTH and stability constant of the complex was 5.86 x 10^{12}. First order derivative technique has been developed. The derivative amplitude was measured by the peak zone method and shows maximum amplitude at 424 nm in acidic buffer medium. The developed first order derivative spectrophotometric method was used for the determination of Cobalt(II) in alloy samples.

Key Words: Cobalt(II), First order spectrophotometry, Bis Vanillin Thiocarbohydrazone(BVTH).

Introduction

The potential analytical applications of hydrazone derivatives have been reviewed by Singh et al [1]. Hydrazones are important class of known analytical reagents [2-7]. In the light of analytical potentialities of hydrazones herein we report the synthesis, characterization and analytical properties of reagent hydrazones.1-Nitroso-2-naphthol proposed by Ilionsky et al [8] was one of the first organic reagents employed for the determination of cobalt. In the light of the above herein we report the derivative spectrophotometric determination of Co (II) using BVTH in alloy samples.

Derivative spectrophotometry is a very useful approach for determining the concentration of single component in mixtures with overlapping spectra as it may eliminate interferences. In this paper a first order derivative spectrophotometric method is described for the determination of Cobalt (II) in alloys.

EXPERIMENTAL

The reagent Bis Vanillin thiocarbohydrazone (BVTH) was prepared by simple condensation of 1 mole of Bis Vanillin with Thiocarbo hydrazone. The structure of the reagent is presented in Fig-1.

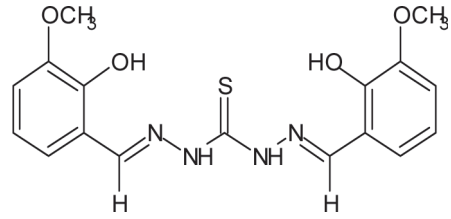

Fig.1: Structure of bis vanillin thiocarbohydrazone

A reagent solution (0.01M) was prepared by dissolving suitable quantity (0.38 g) of the compound in 100 ml of dimethylformamide. The reagent solution is stable for 1 h.

1M hydrochloric acid- 1M sodium acetate(pH 0.5-3.5); 0.2 M acetic acid-0.2M sodium acetate (pH4.0-6.0) and 2M ammonium chloride-2M ammonium hydroxide (pH8.0-11.0) buffer solutions were used in the determination of pKa values of the reagents. The standard cobalt(II) solution (1x10^{-2}M) was prepared using analytical reagent grade cobalt nitrate (AR, BDH). Solutions of diverse ions of suitable concentrations were prepared using AR grade chemicals.

*Address for correspondence:
devanna_nayakanti@gmail.com

Shimadzu 160A UV-Vis spectrophotometer equipped with 1.0 cm quartz cells and an Elico model LI-120 digital pH meter were used in the present study.

Recommended procedure

a) Determination of Cobalt(II) (Zero order): An aliquot of the solution containing 0.0589 to 1.1786 µg/ml of cobalt(II),10 ml of buffer solution (sodium acetate and acetic acid) (pH 6.5) and 1 ml of 0.01 M reagent were taken in a 25 ml volumetric flask and the solution was diluted to the mark with doubly distilled water. The absorbance of the solution aws recorded at 394 nm against the corresponding reagent blank. The measured absorbance was used to compute the amount of cobalt(II) from predetermined calibration plot. It is presented in Fig-2&3.

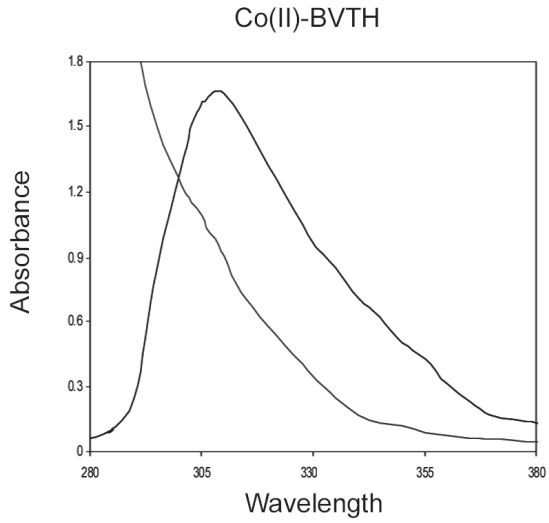

Fig.2: zero order absorbance reagent blank

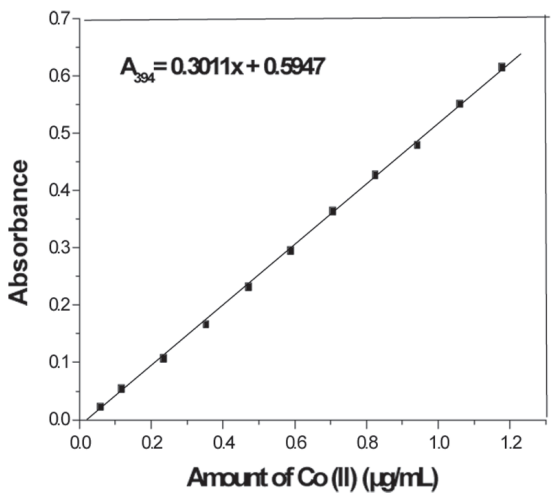

Fig.3: zero order Beer's law spectrum Vs

b) First-order derivative spectrophotometric determination of Co(II): For the above solutions, first-order derivative spectra were recorded in the wavelength range 300-600 nm. The derivative peak height was measured by peak-zero method at 424 nm. The peak height was plotted against the amount of cobalt(II) to obtain the calibration curve. It is presented in Fig-4&5.

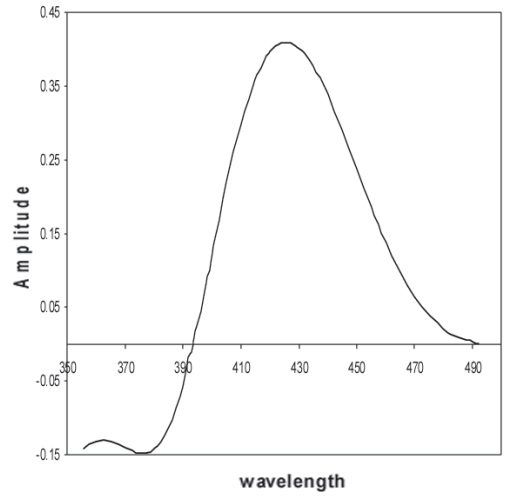

Fig.4: First order derivative spectrum

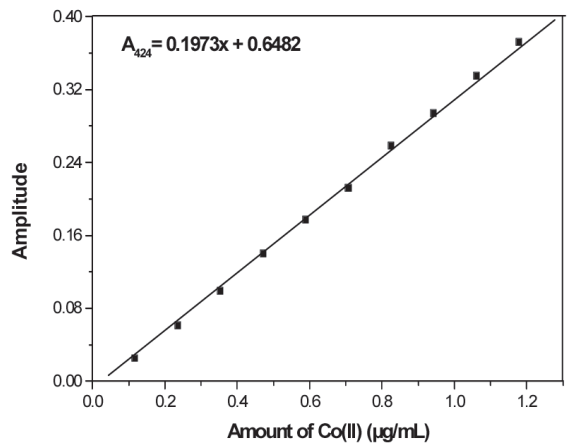

Fig.5: First order derivative Beer's law

Results and Discussion

Bis Vanillin thiocarbohydrazone (BVTH) is a blend of two functional groups, viz., aldehyde and hydrazine. Therefore BVTH may be considered as a two-in-one ligand. This type of reagent is not exploited much for the spectrophotometric determination of metal ions.The pKa values are determined by recording the UV-Vis spectra of micromolar(ca.$1x10^{-4}$M) solution of reagent at various pH values. The complex formation reactions between cobalt(II) with Bis Vanillin thiocarbohydrazone (BVTH) have been studied in detail. The various physico-chemical and analytical properties of Co(II)-BVTH complex are presented in Table-1.

Table.1
Physico-chemical and analytical characteristics of co(II)-BVTH complex

Characteristics	Results
λ_{max}	394 nm
pH range	6.0-7.0
Mole of reagent required per mole of metal ion for full colour development	10 (folds)
Molar absorptivity($L.mol^{-1}cm^{-1}$)	3.2×10^4
Sandell's sensitivity($\mu g/cm^2$)	0.0018
Beer's law validity range(µg/ml)	0.0589-1.1786
Optimum concentration range(µg/ml)	0.2357–1..061
Composition of complex(M:L) obtained in Job's and molar ratio method	1:2
Stability constant of the complex	5.86×10^{12}
Standard deviation in the determination of µg/ml of Co(II) for ten determinations.	0.0005
RSD	0.1

Table 2
Tolerance limit of foreign ions in the determination of 1.1786 µg/ml of Co (II)

Ion added	Tolerance limit (µg/ml)	Ion added	Tolerance limit (µg/ml)
Hypo	3809	Th^{4+}	67
Bromide	2000	Sb^{2+}	51
oxalate	1821	Sn^{2+}	40
Thiourea	1050	Pd^{2+}	34
Urea	1003	Mo^{6+}	28
Acetate	953	Zr^{4+}	26
Thiocyanate	904	Al^{3+}	16
Chloride	773	Hg^{2+}	10
Iodide	570	W^{6+}	4.0
Fluoride	450	$Cu^{2+\dagger}$	4.0
Tartarate	241	Fe^{3+}*	3.0
Phosphate	87	Zn^{2+}	3.0
Citrate	85	V^{5+}	2.0

Interference

Derivative spectrophotometry is a very useful technique in the sense that it decreases the interference, ie., increases the tolerance limit value of foreign ions of metal ions having overlapping spectra. The recommended procedures have been employed for the first-order derivative spectrophotometric determination of cobalt (II).

The effect of various diverse ions in the determination of cobalt (II) was studied to find out the tolerance limit of of foreign ions in the present method. The tolerance limit of foreign ions was taken as the amount of foreign ion required to cause an error of ±% in the absorbance.The tolerance limit values of diverse ions in the determination of µg/ml of Co(II) are found and presented in Table-2.

Applications

Alloy material (0.25 g) was dissolved in concentrated HCl (15 ml) by warming. A little concentrated nitric acid (1 ml) was added and slowly evaporated to dryness. The residue was dissolved in 10 ml of 1M HCl and the resulting solution was concentrated to ca. 5 ml, diluted to ca. 50 ml with distilled water, filtered and made up to 100 ml. Suitable aliquots of the sample were analyzes for the determination of cobalt(II) by following the recommended first-order derivative procedure. The results are presented in Table-3.

Table 3
Determination of Cobalt(II) in Alloy samples

Sample	Cobalt(II)%		
	Certified value	Amount found*	Error (%)
Eligiloy-M-1712	40.00	39.80	-0.50
High speed steel	09.25	09.33	+0.86

*Average of five determinations.

a) Cr 20%; Ni 15%; Co .15%; Fe 15%; Mn 2%; Mo 7%; Fe 0.05%.

b) W 18.5%; Mo 5.5%; Cr 4.15%; Mn 0.40%; Si 0.35%; S 0.05%; rest iron. Iron is masked by fluoride.

Conclusion

From the above discussion, it can be concluded that BVTH is a potential reagent for the derivative spectrophotometric determination of Co(II). it is very easy to synthesize BVTH, a novel class of reagent. The present derivative method is simple and rapid without the need for heating or extraction.

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Optimization of fermentation conditions for bioactive compounds production by river basin *Actinomyceties Streptomyces Kanurubidis*

KANURI TARAKARAM[1], VEDAMURTHY. A.B[2], JAYAVEERA. K.N[3], SIVA SEKHAR. K[4], SATHEESH KUMAR. S[4]

[1]Rural College of Pharmacy, Devanahalli-562110, Karnataka, India.
[2]Oxford College of Science and Research Center, Bangalore, Karnataka, India
[3]Director, OTRI- Jawaharlal Nehru Technological University, Anantapur, Andhra Pradesh 515 001, India.
[4]Center for Bio-Technology, International Science-Tech Research Institute, Anantapur, Andhra Pradesh 515 001, India.

ABSTRACT

13 isolates were isolated from 7 terrestrial soil samples and 5 river sediment samples which were collected through out cauvery river basin, Karnataka. All strains were screened for their potentiality to produce bioactive compounds by using well cut diffusion technique against the following pathogens: *Staphylococcus aureus*, *Streptococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus luteus* and *Candida albicans* as indicator strains. The most potent strain was identified at the molecular level as *Streptomyces Kanurubidis* while the most susceptable strain was *S.aureus*. Well cut diffusion technique was performed using different culture media (nutrient agar, Zobell agar and Luria Bertani), the most suitable medium was Luria Bertani with inhibition zone of 10 mm. Plackett-Burman design was applied to optimize the fermentation conditions and maximize the productivity. The optimized medium was formulated as follows: (g/l): peptone, 15; yeast extract, 2.5; concentrated river water with 1% Nacl (>100%), adjusted to pH 8 and inoculum size 1.5 ml, this medium gives inhibition zone of 16 mm when incubated at 35°C for 48 h i.e. inhibition zone was increased about 1.6 fold increase. Mutation techniques (physical and chemical) were applied to increase bioactive compound productivity but reverse effect was detected. Immobilization using both entrapment (alginate) and adsorption (luffa and pumice) techniques were applied. Only cells adsorbed on pumice gave higher productivity and the inhibition zone reached up to 17 mm.

Keywords: *Actinomyceties Streptomyces Kanurubidis*, optimization, bioactive compounds

Introduction

Bacteria and other micro-organisms are ubiquitous in river basin. They are taxonomically diverse, biologically active, and colonize all environmental habitats, from river sediments to irrigated lands and rocky hilly areas [44]. It has been estimated that the majority of microorganism's natural environmental ecosystems are organized in biofilms [12]. In a biofilm, a microbial community is attached to a surface and embedded in a self-produced matrix composed of extra cellular polymeric substances. This structure provides the microbes present in the biofilm with several advantages compared to those living as plank tonic cells. First, the bacteria are maintained in the selected micro environment where population survival does not depend on rapid multiplication [30]. This is especially advantageous in environments where the microbes are exposed to constant liquid movements, as, for example, in river basin. Additionally, the bacterial cells present in a biofilm have an increased resistance to desiccation, grazing, and antimicrobial agents compared to their plank tonic counterparts [22, 38]. Also, biofilms offer enhanced opportunities for interactions such as horizontal gene transfer and co-metabolism [30, 48].

The occurrence of large scale of bioactive compounds is not common to all living organisms, but restricted to certain taxonomic groups. Recent research progresses reported that many bioactive natural products from soil and river actinomycetes have striking similarities to metabolites of their associated microorganisms including bacteria [43]. Compared with terrestrial organisms, the secondary metabolites produced by river and marine organisms have more novel and unique structures owing to the complex living circumstance and diversity of species, and the bioactivities are much stronger [46].

*Address for correspondence

Competition among microbes for space and nutrient in environment is a powerful selection pressure that endows river basin microbes to produce natural products possessing industrial and medicinal values [3]. Many antimicrobial, antifouling substances have been found among these kinds of organisms due to the specialized role they play in their respective hosts [26]. It is suggested that the primary role of these antibiotic substances could be related to ecological competition [8].

The nutritional and environmental conditions have a great influence on production of the antimicrobial substances [34]. In order to develop an efficient production of antimicrobial substances, knowledge regarding the environmental factors affecting this process needs to be well identified. Experimental designs are excellent techniques for optimization of culture conditions to achieve optimal production [14, 15].

The aim of this work was isolation of some river basin actinomyceties capable of producing bioactive compounds and optimizing the fermentation conditions for maximum production.

Materials And Methods

Organisms and Maintenance: 13 isolates were isolated in cauvery river basin from 7 terrestrial soil and 5 river sediment samples. Which were collected on nutrient agar medium. All isolates were screened for their potentiality to produce bioactive compounds against the pathogen *Staphylococcus aureus* (NCIM 6538), *Streptococcus faecalis* (NCIM 8043),

Pseudomonas aeruginosa (NCIM 8739), *Escherichia coli* (NCIM 8739), *Micrococcus luteus* (NCIM 10240) and *Candida albicans* were used as indicator strains. These indicators were kindly provided by NCIM, National Chemical laboratories, Pune, India. The most promising isolate was chosen and identified according to the standard procedures described by [47, 49, 52]. Moreover it was subjected to molecular identification.

Antagonistic Action against Indicator

Microorganisms: The well-cut diffusion technique was used to test the ability of the isolates to inhibit the growth of indicator bacteria and yeast, 50¼l was add in each well, After incubation period, the radius of clear zone around each well (Y) and the radius of the well (X) were linearly measured in mm, where dividing Y^2 over X^2 determines an absolute unit (AU) for the clear zone. The absolute unit of each antagonistic isolate, which indicates a positive result in the antagonistic action, was calculated according to the following equation [16]: $AU= Y^2/X^2$. All experiments were done in triplicates and the average was calculated.

Molecular Characterization:

Isolation of S IV-05 DNA: The genomic DNA of the most promising producer S IV-05 was isolated from overnight cultures according to the method described by Sambrook *et al*. 1989. Cells were collected by centrifugation and re-suspended in 500 µl TEN buffer. After incubation at 37°C for 30 min, 30 µl of 10% Sodium Dodecyl Sulphate SDS were added and the tubes were inverted gently several times till complete lyses. An aliquot of 5 mg/ml of proteinase K added and the tubes were incubated at 37°C for one hour. After incubation the solution was phenol-extracted several times to remove the protein and once with chloroform to remove the phenol traces. The DNA was precipitated using 0.8 volume of isopropanol and washed with 70% ethanol. The DNA was dried and dissolved in 10 mM Tris HCl, pH 8 and stored at 20°C.

Amplification of 16S rRNA Gene: The 16S rDNA was amplified by polymerase chain reaction (PCR) using the primers 16F27 and 16R1492. Approximately a 1500-base pair fragment of 16S rDNA region was amplified according to the *Escherichia coli* genomic DNA sequence. Amplicons were obtained with a PCR cycling program of 94°C for 1 min followed by 30 cycles of denaturating at 94°C for 1 min, annealing at 55°C for 1 min and polymerization at 72^0 for 2 min. At the end of thermo cycling, The PCR reaction mixture was incubated at 72°C for 10 minutes. As described by [4]. Amplicons were visualized by electrophoretic separation on 1% agarose gels stained with ethidium bromide. PCR fragments were purified from amplification reactions with QIA quick PCR purification reagents (QIA EN) according to the kit manual.

Sequencing of PCR-DNA Product: DNA sequence was obtained using DNA sequencer (ABI 310). The PCR product was sequenced using the same PCR primers and other internal primers to confirm the sequence.

Sequence Similarities and Phylogenetic Analysis: Blast program (www.ncbi.nlm.nih.gov/blast) was used to asses the DNA similarities. Multiple sequence alignment and molecular phylogeny were performed using BioEdit software [23]. The phylogenetic tree was displayed using the TREEVIEW program [41].

Effect of Culture Medium Type on Production of the Bioactive Compounds: The selected isolate S IV-05 Strain was grown using nutrient agar, ZoBell and Luria Bertani (LB) media (prepared with river water) at 30°C for 24 hrs. Culture cell free supernatant was tested against the previously chosen indicator organisms using agar well diffusion technique aiming to obtain the highest productivity [1].

Evaluation of Nutritional Factors: The Plackett-Burman design [42, 53] was applied to reflect the relative importance of various parameters involved in the production of these bioactive compounds by the chosen isolate S IV-05. For each variable a high (+) and low (-) levels were tested. The examined variables in this experiment and their levels are shown in Table 2. Eight different trials were performed in duplicates. Rows in Table 3 represent the different trials (row no. 9 represents the basal control). The main effect of

each variable was determined with the following equation:

$$Exi = (Mi+ - Mi-) / N$$

Where Exi is the variable main effect, and Mi+, Mi- are the radius of the clear zone around each well in the trials, where the independent variable was present in high and low concentrations, respectively, and N is the number of trials divided by 2. Statistical t-values for equal unpaired samples were calculated using Microsoft Excel to determine the variable Significance.

Effect of Mutations on Bioactive Compounds

Production: To study the effect of mutations on the productivity of the selected S IV-05 Strain against the different pathogens, physical mutation was performed using UV light (254 nm) at different time intervals in order to plot the survival curve. Induction of mutation was carried out at a dose that yielded 90% mortality as determined from the previous survival curve following the procedure of Kung & Lee [33]. To study the effect of chemical mutation, ethidium bromide was used as chemical mutagen according to [21]. Each mutant was tested for its potent ability against the previously mentioned pathogens using the well cut diffusion technique.

Effect of Immobilization on Bioactive Compounds Production: Immobilization was performed using both entrapment and adsorption techniques as was described by Eikmeier & Rehm, [13] aiming to enhancing the production of the bioactive compounds against different pathogens.

Electron Microscopy: The adhesion of the selected S IV-05 on or in the supporting materials and formation of the biofilm was carried out using scanning electron microscope. Cells grown in LB were harvested by centrifugation, washed with phosphate buffer and fixed with 2% glutaraldehyde followed by 1 % osmium tetroxide treatment. After complete fixation, samples were washed in buffer solution, and then dehydrated in ascending order of ethanol concentrations. The samples were dried completely in a critical point dryer, and finally coated with gold in JEOL-JFG1100 E ion sputter-coater. The specimens were viewed in JEOLJSM 5300 microscope operated at 20 kV with a beam specimen angle of 45^0.

Results And Discussion

Results: The isolate S IV-05 was the most promising strain, it inhibited the growth of all pathogens especially *Staphylococcus aureus*, the inhibition zone was (9mm) on river water agar medium. This strain was isolated from the surface of the terrestrial soil of cauvery basin. It is biochemically identified as *streptomyces sp*. This was confirmed using the molecular techniques.

Molecular Characterization of *streptomyces* Species:

DNA of the promising *streptomyces* sp. was extracted and the extracted 16S rRNA gene was amplified using the universal primers 16F27 and 16R1492. The produced amplicons was analyzed using agarose gel electrophoresis as shown in Figure 1. It was clear that this strain showed nucleotide size of 1490 base pair compared with phage λ DNA *Hind* III cut molecular weight marker.

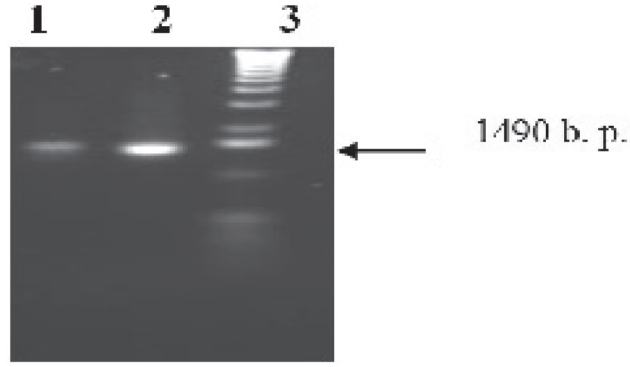

Fig. 1: 16S agarose gel electrophoreses of the extracted and amplified DNA. Lanes1& 2= purified PCR products of the isolate, and lane 3 is λ phage DNA Hind III cut molecular weight marker.

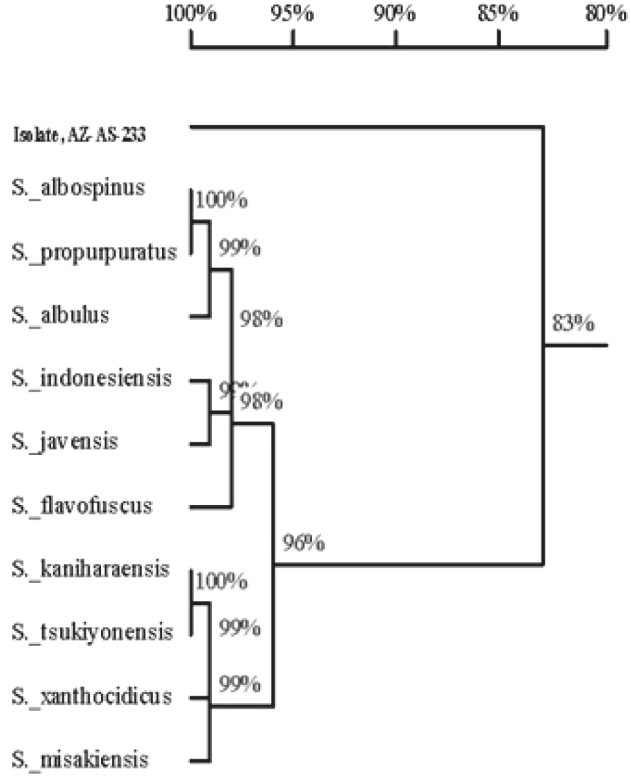

Fig 2: phylogenetic relationships among representative experimental strain and the most closely related streptomyces species. The dendogram was generated using Tree View Program

Sequencing of PCR-DNA Products: The amplified DNA was sequenced using ABI 310. The sequencing data obtained utilizing this strategy was 500 base pair which represents the partial coding sequence of 16S rRNA gene. The sequencing data was analyzed using nucleotide BLAST search computer based program where this sequence was compared with that of any other rRNA (or rDNA) genes that have been sequenced so far. The resulting data indicated that the isolate under study was identified as *Streptomyces Kanurubidis* with identity percentage 92% which confirms the identification using the traditional biochemical tests. The phylogenetic tree was displayed using the TREEVIEW program as shown in Figure 2.

Optimization of Bioactive Compounds Production by *Streptomyces Kanurubidis:*

Effect of Medium Type on the Production of the Bioactive Compounds: *Streptomyces Kanurubidis* was examined for the antagonistic activity using three different media; nutrient agar (NA), Luria Bertani (LB) and ZoBell river water agar . As shown in Table 1, the highest activity was observed using LB.

Table 1
Effect of the growth media on the production of the bioactive compounds by *S. Kanurubidis*

Inhibition zone (mm) produced by the selectedstrain using different media		
LB	NA	ZoBell
10	9	9

Table 2
Independent variables affecting production of the bioactive compounds and their levels in the Plackett-Burman design

parameters	symbols	Level		
		-1	0	1
Peptone(g/l)	P	5	10	15
Yeast extract (g/l)	Y	2.5	5	7.5
Inoculum's size (ml)	IS	0.5	1	1.5
River water with 1%Nacl	C	50%	100%	>100%
pH	pH	6	7	8
Temperature (^{0}C)	T	25	30	35
Incubation period(h)	IP	12	24	48

Optimization of the Fermentation parameters: The Plackett-Burman design was applied to reflect the relative importance of various parameters involved in the production of these agents by *S.Kanurubidis.* The main effect of each variable on the production of the bioactive compounds as well as *t-values* were estimated for each independent variable as shown in Table 4 and graphically presented in Figure 3. Results in this Figure indicated that the main effect of all variables were positive on the production by *S. Kanurubidis* except for yeast extract where high concentration of yeast extract in the medium causes decrease in the production. Statistical analyses of the results (t-test) showed that variations in yeast extract and the incubation period in the tested ranges had the most considerable effects on the production of bioactive compounds by *S. Kanurubidis.*

Table 3
Experimental results of the Plackett- Burman design

Trials	Parameters symbols							Diameter of inhibition zone
	Y	P	IS	C	pH	T	IP	mm
1	-1	-1	-1	1	1	1	-1	11
2	1	-1	-1	-1	-1	1	1	16
3	-1	1	-1	-1	1	-1	1	0
4	1	1	-1	1	-1	-1	-1	0
5	-1	-1	1	1	1	-1	1	14
6	1	-1	1	-1	-1	-1	-1	12
7	-1	1	1	-1	1	1	-1	0
8	1	1	1	1	-1	1	1	15
9	0	0	0	0	0	0	0	10

Table 4
Statistical analysis of the Plackett-Burman experimental design

Variable	Main Effect	*t-value*
Peptone	4.75	0.8
Yeast extract	-9.75	-2.4
Inoculums size	1.75	0.36
River water with 1% Nacl	3.25	0.6
pH	2.75	0.55
Temperature	4.25	0.76
Incubation period	5.75	1.09

t-value significant at the 1% level = 3.70

t-value significant at the 5% level = 2.45

t-value significant at the 10% level = 1.94

t-value significant at the 20% level = 1.37

Standard *t -values* are obtained from Statistical Methods (Cochran and Snedecor, 1989).

The interacting effect of yeast extract with the incubation period is described in three-dimensional representation (Figure 4). As illustrated, the inhibitory effect of high levels of yeast extract on the antagonistic activity of *S. Kanurubidis* can be partially overcome by preparing cultures with decreasing level of yeast extract and extending the incubation period.

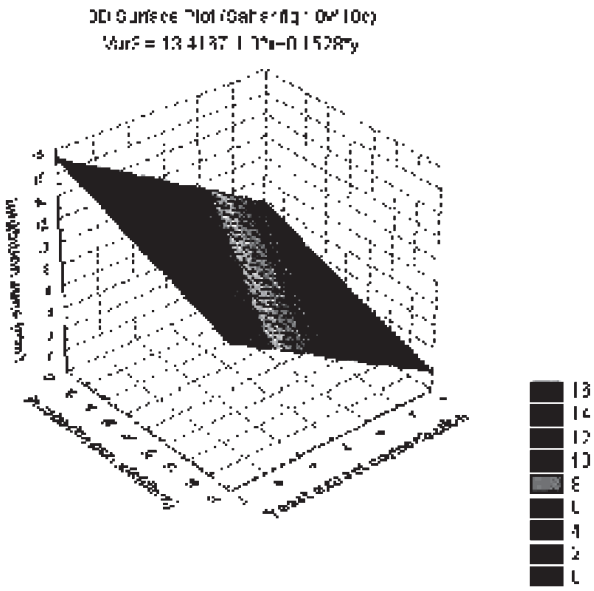

Fig. 4: The interaction effect of yeast extracts concentrations (g/l) with incubation periods (days) Levels, with respect to inhibition zone (mm) based on Plackett-Burman results.

According to the obtained results, the predicted medium for cultivation of *S. Kanurubidis* to enhance maximum production of the bioactive compounds was formulated as follows: (g / l): peptone, 15; yeast extract, 2.5; concentrated river water with 1% Nacl (>100%) , adjusted to pH 8 and inoculums size (1.5 ml for each 50ml medium) all of which are incubated for 48 h at 35°C.

In order to evaluate the accuracy of the applied Plackett Burman statistical design, a verification experiment was applied to compare between the predicted near optimum levels of independent variables and the basal condition settings. Results in Table 5, confirmed that, the production of the bioactive compounds increased and the inhibition zone increased by 1.6 fold increase.

Table 5
A verification experiment showing antagonistic activity of *S. Kanurubidis* grown on basal against optimized media

Inhibition zone (mm)	Basal medium	optimized medium
	10	16
Activity unit (AU/ml)	4	10.24

Effect of Mutation on Bioactive Compounds Production: *S.Kanurubidis* was subjected to two types of mutations (physical and chemical) to investigate the productivity of bioactive compounds. It was subjected to irradiation with UV lamp at 254 nm as a physical mutagen and ethidium bromide as a chemical mutagen. Variants obtained from both types of mutation were tested for the production of bioactive compounds to select the most potent one.

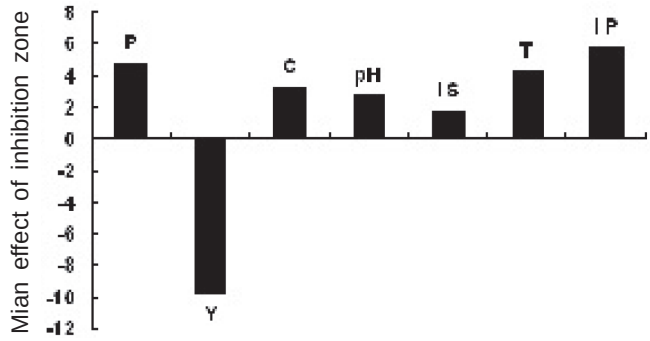

Fig. 3: Elucidation of fermentation conditions affecting the production of the antagonistic agents of *S. Kanurubidis.*

Concerning the physical mutation by UV irradiation, a survival curve was plotted using survival %of the treated strains against the exposure time (h) as shown in Figure 5. Exposure time at which about 90% lethality of the bacterial population was 1 min. Capacity of bioactive compounds production was determined for each mutant. Comparison between the wild type and its variants is presented in Table 6. Results indicated that UV mutant of *S. Kanurubidis* exhibited lower activity (13 mm inhibition zone) compared to its wild type (16 mm) while ethidium bromide-mutant was better than UV-mutant (15 mm) but still lower than the activity of the wild type.

Table 6:
Comparison among the wild type and their mutants for production of the bioactive compounds

S. Kanurubidies	Wild type	Mutant (UV)	Mutant (ET)
Inhibition zone (mm)	16.0	13.0	15.0
Activity unit (AU/ml)	10.24	6.76	9.0

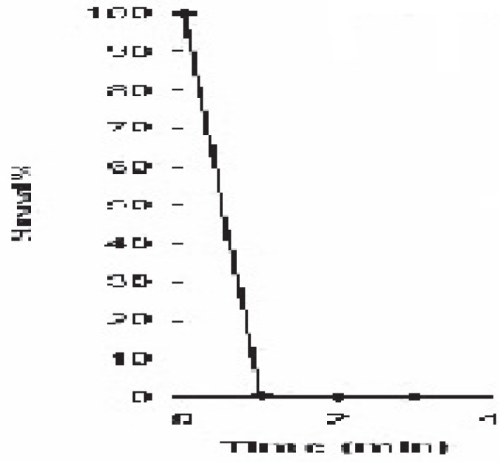

Fig. 5: Survival curves of *S. Kanurubidis* treated with UV irradiation.

Effect of Immobilization on Bioactive Compounds Production by *S.Kanurubidis:* Living cells of *S. Kanurubidis* were subjected to immobilization using adsorption and entrapment techniques. Adsorption was carried out using both luffa pulp and pumice as supporting materials. Figure 6 showed the adsorption of the cells on luffa pulp and pumice while entrapment was done using sodium alginate as a gel matrix. The aim of this experiment is to compare the production of antimicrobial agents by both the free and immobilized cells of *S. Kanurubidis.* The optimized medium containing the adsorbed cells of *S. Kanurubidis* was used. Results in Table 7 revealed that *S. Kanurubidis* on pumice showed relatively higher activity in the production of the bioactive compounds (1.2 fold increase) in the diameter of the inhibition zone.

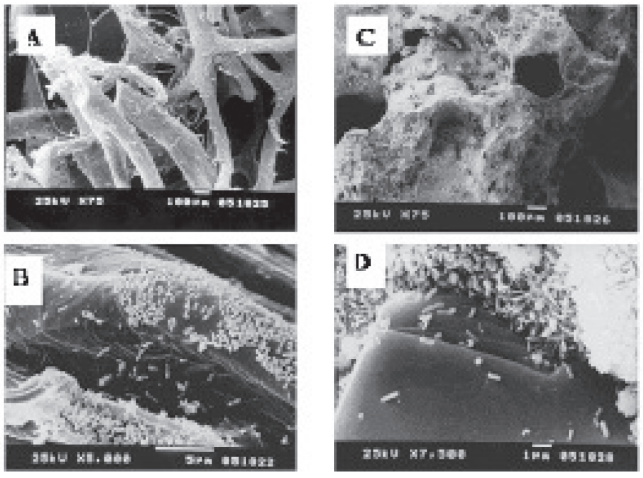

Fig. 6: Scanning electron micrographs showing (A) control luffa pulp; (B) the wild *S. Kanurubidis* adsorbed on luffa pulp; (C) control pumice and (D) the wild *S. Kanurubidis.*

Using luffa pulp as the supporting material decreased the production to about 0.81 fold in the inhibition zone and about 0.66 fold in the activity unit compared to the free non-fixed cells. By using entrapped cells of the wild *S. Kanurubidis*. Results indicated a decrease in the inhibition zone diameter to about 0.75 fold and about 0.56 in the activity unit compared to the free cells.

Discussion:

River organisms are a rich source of structurally novel and biologically active metabolites [7, 18,]. Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry [20]. To date, many chemically unique compounds of marine origin with various biological activities have been isolated, and some of them are under investigation and are being used to develop new pharmaceuticals [20, 35]. The ability of river basin Actinomycetes to produce secondary metabolites of potential interest has been extensively documented.

The most promising isolate S IV-05 (*S. Kanurubidis*) identified using molecular techniques. Recently, 16S rRNA sequence comparison has been used as a powerful tool for establishing phylogenetic and evolutionary relationships among organisms [37]. An approach presently employed in many laboratories uses the polymerase chain reaction [25, 51] to obtain 16S rRNA-specific genes for sequence analysis. Sequencing of PCR-product and comparison of 16S rRNA (rDNA) sequences has been reported among type strains of *Streptomyces* [24, 6, 51].

The phylogenetic relationships among the new experimental isolate S IV-05 and the closely related *Streptomyces* species have been described in the present work and revealed that, strain S IV-05 was taxonomically positioned within the *S. Kanurubidis* group representing 92% identity. The data obtained by 16S rRNA coincide with those found by traditional, morphological, physiological and biochemical methods. This strain was identified as *S. Kanurubidis*

Different type cultures were tested for their sensitivity to the bioactive compounds produced by *S. Kanurubidis* to achieve the highest antagonistic activity.

S. Kanurubidis was grown in Nutrient agar, ZoBell and LB media and was tested for the antagonistic effect against S. aureus. LB was found to be the most suitable medium to achieve the highest antagonistic activity as was reported by [1].

In the present study, Plackett-Burman design was employed which was successfully employed in enzyme production and other optimization experiments [25, 51, 2]. Results revealed that concentration of yeast is the main factor affecting the activity of the bioactive compounds produced by S. Kanurubidis where it was significant at 1% level. Decreasing the levels of this factor yielded the highest

Table 7

Effect of immobilization on production of the bioactive compounds by the *S. Kanurubidis* cells

Bacterial strains	Antagonistic activity using different support materials							
	Control		Luffa pulp		Pumice		Ca-alginate beads	
Wild *S.Kanurubidis*	IZ(mm) 16	AU/ml 10.24	IZ(mm) 13	AU/ml 6.76	IZ(mm) 17	AU/ml 11.56	IZ(mm) 12	AU/ml 5.76

antagonistic effect against the tested pathogen.

It was noticed that the highest production of the antimicrobial agent by S. Kanurubidis was obtained at pH 8 [17]. Reported that pH 8 was the optimum pH for the maximum production of bioactive compounds from Nocardia brasiliansis.

Leal– Sánchez, [34] reported that temperature was found to have positive significant effects (p#5) on the production of the bioactive compounds which is in agreement with our study. In the present study, river water concentration exhibited positive effect on the production by S. Kanurubidis as shown by [36]. Inoculum size had also positive effect on the production [2].

Another study carried out by [28] stated that fermentation time is very important optimizing parameter. In this study, the maximal production of the bio-active compounds was obtained by S. Kanurubidis after 48 h after which, the activity decreased significantly in the culture medium. Similar results were also reported by [10].

It was shown that increasing the incubation period, peptone, inoculum size, temperature, pH and river water concentration have positive effects on the production of bioactive compounds by S. Kanurubidis. Therefore, results concluded that to achieve the highest antagonistic effect by S. Kanurubidis, the medium composition should be: (g/l) peptone , 15; yeas t extract, 2.5; river water with 1% Nacl concentration (<100%), pH 8 with inoculum size 1.5 ml (for each 50 ml medium) for 48 hrs at 35°C. Under such conditions, th e activity unit produced by S. Kanurubidis showed 10.24 AU/ml (2.56 fold increase) than that obtained using the basal growth medium.

The effects of mutations on the production by different bacterial strains have been reported but in our study, the mutations decreased the production of bioactive compounds.

Immobilization on pumice showed (1.13) fold increase in the productivity compared t o the free cells while immobilization on luffa and inside the beads of sodium alginate reduces the productivity and these results may be due to one or more of the following reasons: the support may possesses poor mechanical stability, as reported by Klooster and Lilly, [32]. Diffusion limitation is a second important factor where substrate limitation/product inhibition in entrapped immobilization systems may also affect this process [5]. A third factor is the individual characteristics of the bacterium [31]. Where Ivanova et al., 1998 stated that the antibiotic production of 12 strains of epibiotic bacteria was enhanced after immobilization.

We can conclude that river basin actinomycetes S. kanurubidis have the potentiality to produce highly effective bioactive compounds which must be applied in aquaculture or in the production of pharmaceutical agents.

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Simultaneous determination of lead and zinc using newly synthesized analytical reagent 4-(2-hydroxy phenyl ethaminodiol), benzene-1.3-diol by simple chloroform extraction procedure prior to anodic stripping voltammetric analysis.

[A]*SREEDHAR. N.Y, REKHA. D[A] AND SANKAR NAYAK. M
[a]*Department of chemistry, S.V.University, Tirupati 517502 A.P, India

ABSTRACT

Analysis of metal pollution is the major drastic approach for the chemist who is putting their efforts to protest the metal pollution which is arising from so many deeds by human beings and industrialization etc. Hence author reported an analytical method for the determination of trace metals in pharmaceutical and biological samples. The simultaneous determination of lead and zinc by anodic stripping volammetry after extraction with chloroform is described. For this purpose a novel and newly synthesized analytical reagent 4-(2-hydroxy phenyl ethaminodiol), benzene-1.3-diol (4-2-HPEDB-1,3,D) was used for the metal complexation. This paper out lines the electrochemical behaviour of metal complex in pharmaceutical preparation and biological samples. The calibration graph was constructed in the concentration range of 0.001-200 μg/l at pH 4.5 (acetate buffer) having the correlation coefficient of factor of 0.9998 for lead, 0.9995 for zinc and RSD will found to be 5.3 %. The study of interfering ions during the analysis of lead and zinc with the error of >2%, shows the sensitivity and selectivity of the proposed method. The effect of pH, effect of pulse amplitude, scan rate and other analytical parameters were optimized. The stoichiometry of the complex was estimated as 1:1 ratio by molar ratio method and Job's continuous variation method. The proposed method is successfully applied for the determination of lead and Zinc in biological samples and the results obtained were shows the good accuracy and precision with the reported method which is exist in literature.

Keywords: Lead, Zinc, Extraction procedure, anodic stripping voltammetric, biological samples.

Introduction

Now days environment getting more pollution due to release of so many heavy metals which has considerable attention , due this more and more efforts have been put to remove the toxic metal from different from different fields of environment .according t this lead and zinc posses high toxic potential which are accumulated in living organisms and environmental samples. Metals are tend to accumulate the biologically active region of the soil, where they can be taken up by crops.

Lead is soft malleable and stable metal and it is chemically similar to calcium and therefore it is accumulate bone matrix. Lead is highly toxic to human beings and animals even low at concentration. It involves the biosynthesis and affects the kidney, liver membrane, brain cells. When it accumulate in the body it causes some diseases like nausea, vomiting, diarrhea, sweating, convulsions, coma and some times finally it leads to the death. Apart from that the major use of lead is battery production, it is used in chemical industry for preparation of paints, pigments and colored inks. The main source of lead is lead mines, smelters and battery production.

Zinc plays an important role in exerting beneficial effects on cardio circulatory function and in prevention of black foot disease [1]. It is essential element for exceptional biology and public health and its deficiency causes growth retardation, delayed sexual maturation, infection susceptibility and diarrhea. Excessive absorption of zinc may lead the suppression of copper and iron absorption in to living organisms. Application of zinc is used as catalyst in rubber manufacturing. Zinc mainly acts as antioxidant.

Several analytical techniques like spectrophotometry [2], AAS [3-5], NAA [6], ICPAES [7], ICPMS [8],

*Address for correspondence:
sreedhar_ny@rediffmail.com

Chromatography [9] have been reported for the determination of zinc and lead but the above said techniques are expensive and required special laboratory conditions for analysis. In addition to that it requires time consuming and tedious reaction process.

In view this it is very important to develop an analytical technique for the monitoring of lead and zinc in different environmental samples. For this purpose an alternative method for the analysis of metals ions.

In the present study author reported an electroanalytical technique anodic striping voltammetry using newly sysnthesised reagent 4-(2-hydroxy phenyl ethaminodiol), benzene-1.3-diol (4-2-HPEDB-1,3,D) for the complexation of metal ion. A simple extraction procedure was developed before injecting the sample in to instrument. For this process author selected organic solvent chloroform as metal complex extracting agent which is high dielectric constant. The proposed method is applied for the determination of lead and zinc in pharmaceutical preparation and biological samples.

Experimental

Apparatus

Instrument used for analysis by anodic stripping voltammetry in differential pulse mode was an Elico CL-362 model polarographic system and Elico Li-129 Model glass-calomel combined electrode was employed for measuring pH values.

Three electrode systems consisting of hanging mercury drop as working electrode, Ag/AgCl (salt KCl) was used as a reference electrode which provide a reversible half reaction with nernstian behavior be constant over time and easy to assemble and maintain and a platinum wire as an auxiliary electrode which displays negative potential range.

Reagents

All reagents used were of analytical reagent grade. Double distilled water was used throughout the experiment. A stock solution of lead (II) and Zinc (II) was prepared by dissolving appropriate amount of their respective salts in double distilled water in volumetric flask. Working standard solution was freshly prepared by diluting the stock solution with double distilled water. 0.1 M concentration of 4-2-HPEDB-1,3,D was prepared by dissolving 2.48 g of 4-2-HPEDB-1,3,D in 100 ml of methanol.

Synthesis of 4-2-hydroxy phenyl ethaminodiol benzene-1,3-diol (4-2-HPEDB-1,3,D)

Equimolar ratio of 2,4-dihydroxy acetophenone and 2-aminophenol in methanol mixture was refluxed for 3-4 hours and the contents were cooled at room temperature, it gives orange-red colour precipitate. The precipitate was filtered and washed with methanol to pure Schiff base. (M.P-115 ^{0}C, yield 97%) as shown in Scheme I.

I.R –(KBr), 1601.8cm$^-$(C=N), 3304.0 cm$^-$ (N-H), Free (OH) 3375.3 cm$^-$ 1465.9 cm$^-$(O-H) and the spectrum was shown in fig 1.

Recommended analytical Procedure for the determination of metal ions

An aliquot of working standard solution containing 1-100 µl of metal ion is taken in to 25 mL volumetric flask. To this 5 ml of acetate buffer solution (pH 4.5), 2ml of reagent solution were added. The mixture was shaken with 5.0 mL portion of chloroform for 30s and allowed to stand for 5-10 min. The organic phases curve collected and tranfered in to electrolytictic cell and diluted with 9 ml of supporting electrolyte and then deoxygenated with nitrogen gas for ten min. A fresh drop of mercury of known area extrucded from the micrometer of the hanging mercury drop electrode (HMDE) and the solution was stirred at constant and reproducible rate come out disturb the mercury drop . The two elements were determined in the ASV mode. Electrolysis was done at -0.80 V Vs SCE deposition time 5 min, pulse amplitude of 50 mV, scan rate 2mVs^{-1} and pH 4.0 for all two elements. The maximum peaks appearing for sample is at -0.56V, -70V for Lead and Zinc respectively which shown in fig 2.

Collection of samples

The samples were collected from different locations of the study areas in and around Tirupati. The necessary and possible precautions were taken at various stages starting from sample containers, sample collection and storage, processing and analyzing the samples.

Analysis of biological samples

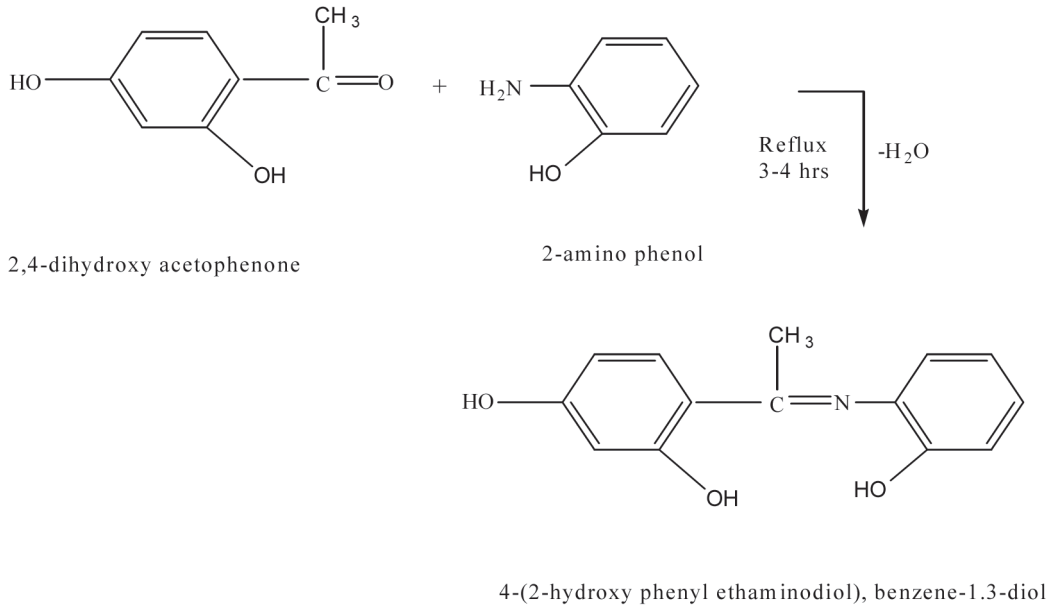
The hair samples were washed with acetone 2-3 times in a beaker with continuous stirring. Then they were dried in an electric oven at 70° C for 4 h. Two grams of the sample was weighed and taken in a beaker. To this a (1:1) mixture of nitric acid and perchloric acid was added, and the mixture was heated on a hot plate. The solution was evaporated to near dryness. The ash was taken up with 5 mL of HCl (1+9) and evaporated to dryness. The residue was taken up in 2 ml conc. HCI, filtered and made up to 25 mL with water. Suitable volumes of these solutions are taken for the determination of lead and zinc as described in above said procedure and the results were shown in Table 1.

Result and discussion

Voltammetric studies

Effect of pH

The effect of pH on the peak potential E_p and current intensity i_p, using voltammogram was examined for [M-(4-2-HPEDB-1,3,D)]. Where (M=Pb, Zn) The pH was varied in the whole pH range 2.0 to 10.0 for [M-(4-2-HPEDB-1,3,D)] complex. It can be observed from Figure 2, - 56.0 mV, -70.0mV that the maximum peak current obtained



Scheme I: Synthesis of 4-(2-hydroxy phenyl ethaminodiol) benzene-1.3-diol (4-2-HPEDB-1,3,D)

Table 1
Determination of lead and Zinc in biological samples

Samples	Certified values		Present method	
	lead	Zinc	lead	Zinc
1.Human serum (GBW 09135)	0.17	1.03	0.172±0.06	1.05±0.7
2.Human hair (GBW 09101)	7.2	189	7.25±0.19	191±0.8
3.Bovine liver(CZIM-LINER)	0.71	162	0.73±0.02	164±0.9

Table 2
Study of Interfering ions during the analysis of lead and zinc

Interfering species	Tolerance limit for lead	Tolerance limit for Zinc
K^+, Na^+, Cs^+, HCO_3^-, I^-, Br^- , Cl^-, N^{-3}, ClO^- 4 ClO_2^{-3} NO^{-2}, F^-, IO, BrO, Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn(II), Al^{3+}	1000	1000
Zn^{2+}	1000	-
Pb^{2+}	500	1000
Sr^{2+}	500	500
Rh(III)	250	20
CrO	250	100
Co^{2+}	250	50
Ce(IV)	250	50
CN^-	100	50
Cu^{2+}, Ni^{2+}, Fe(III) 50	50	50
Pd(II)	20	250
Fe(II), Hg(II)	20	50

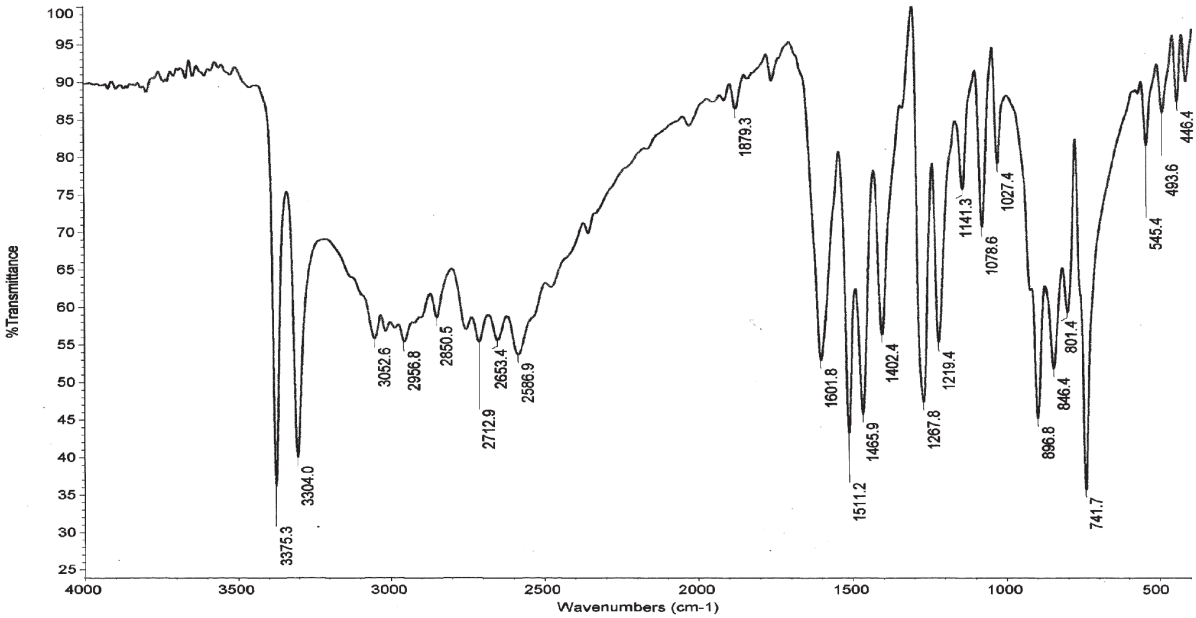


Fig. 1: I.R spectrum for 4-(2-hydroxy phenyl ethaminodiol) benzene-1.3-diol (4-2- HPEDB-1,3,D)

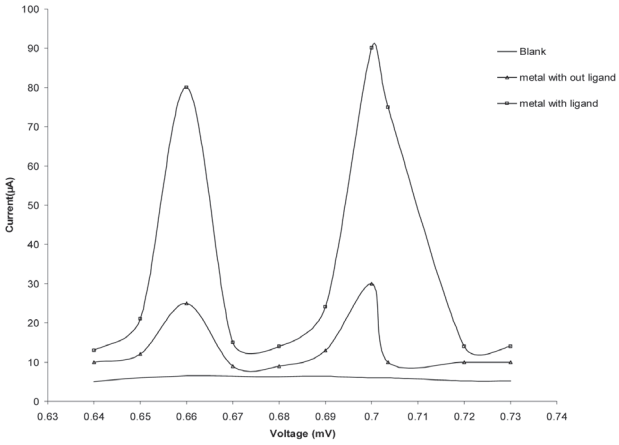


Fig. 2: Differential pulse polarogram of Pb and Zn (II). Peak at -56.0 mV and -70.0mV

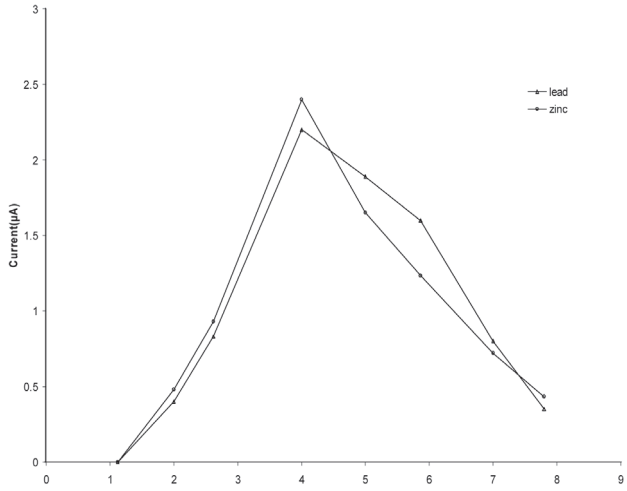


Fig 3. Effect of pH on determination of Pb and Zn .

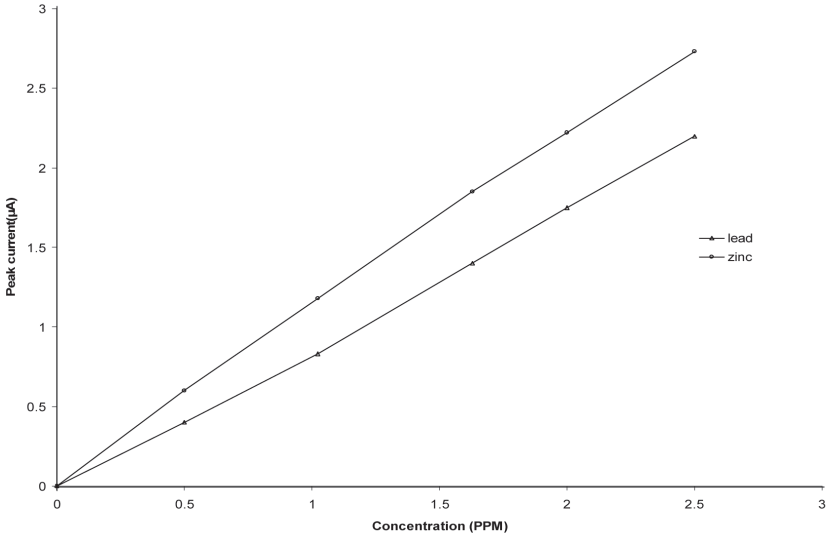


Fig. 4: Calibration curve for Pb and Zn

with pH 4.0. When the pH has been increased from 2.0 to 10.0 the peak potentials have been shifted towards more negative values, indicating proton participation in the reduction process and the results were shown in fig 3.

Effect of Scan Rate

As for the scan rate; the current response with increasing the scan rate of 40 mVs^{-1} gave the maximum response. Accordingly, the optimum conditions for recording a maximum developed and sharper voltammetry peak for 0.5 mM [M-(4-2-HPEDB-1,3,D)] are scan rate: 40mVs^{-1} and pulse amplitude : 50 mV.

Other experimental parameters such as temperature and ionic strength were optimized. The stripping peak currents were not modified when the temperature varied between 20-50°C. The value chosen was 25°C.

Effect of deposition potential

It is generally recognized that the Pb and Zn accumulate on the mercury electrode in the anodic potential range as results of mercury oxidation followed by the formation of amalgam. Consequently it is expected that strong dependence of the accumulation efficiency on the factor determining the state of the electrode solution interface would be observed at deposition potential -80 V vs SCE maximum current is observed for the two elements.

Effect of deposition time

The peak current increased markedly with increasing accumulation time. For accumulation time higher than 5 times the shape of voltammogramms of Pb and Zn are changed and an addition peak is observed due to the reduction of amalgam.

Effect of Solvent

The extraction of [M-(4-2-HPEDB-1,3,D)] complex was carried out with different organic solvents like dimethyl formaldehyde, CCl_4, Cyclohexane, chloroform, xylene, tolunene, n-butanol, 1-pentanol, 1-amyl alcohol and nitrobenzene. Among these solvents the extraction of [M-(4-2-HPEDB-1,3,D)] complex efficiency more in chloroform when compare to other organic solvents. Therefore chloroform is chosen as solvent for extraction of [M-(4-2-HPEDB-1,3,D)] complex for further studies.

Stoichiometry of the complex

The composition of the complex was found to be 1:1 = M^{2+}: 4-2-HPEDB-1,3,D . The Stoichiometry of the complex was verified by mole ratio method.

Calibration

The calibration curve was constructed based on the general procedure under the optimised conditions having the concentration range of 0.01- 200 µg/m1 with correlation coefficient found as 0.9998 for lead and 0.9995 for zinc. The relative standard deviation as 5.3% and it's relative typical curve was shown in fig 4.

Effect of Foreign ions

The Study of the interfering ions during the analysis of lead and zinc in pharmaceutical preparations biological samples was shows the sensitivity and selectivity of the present method and the results were shown in Table 2. The metal ions are individually added to the lead and zinc ions having appropriate concentration and the general procedure was applied. The tolerable limits of various foreign ions are masked using suitable masking agents and recovery ranges with the error (<2%) are shown in Table 2.The results are almost quantitative in the presence of interfering ions to evaluate the feasibility of the present method.

Conclusion

The present method for the simultaneous analysis of the lead and zinc using chloroform extraction procedure by anodic striping voltammetry is an alternative method for lead and zinc determination at hanging mercury drop electrode. A new analytical reagent 4-(2-hydroxy phenyl ethaminodiol), benzene-1.3-diol (4-2-HPEDB-1,3,D) was synthesised at ordinary laboratory conditions which is economically very cheep and simple for the determination of lead and zinc. The accuracy and precision of the present method was checked by the certified reference material and the statistical data which is obtained from the proposed is good agreement with certified materials. The present method is successfully applied for the determination of lead and zinc in biological samples.

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Phytochemical, Antibacterial and Anthelmintic potential of flowers of *Polyalthia longifolia*

*JAYAVEERA. K.N[2], SRIDHAR.C[4], KUMANAN. R[1], YOGANANDA REDDY. K[1], TARAKARAM. K[3], MAHESH. M[1]

*[1]Center for Natural Product and Herbal Medicines, International Science-Tech Research Institute, Anantapur, Andhra Pradesh-515001

[2]Director, JNTU-OTRI campus, Jawaharlal Nehru Technological University Anantapur - 515001, Andhra Pradesh.

[3]Principal, Rural College of Pharmacy, Devanahalli, Bangalore 562110, Karnataka.

[4]CES College of Pharmacy, Kurnool, Andhra Pradesh.

ABSTRACT

The present study evaluated the antibacterial potentiality; phytochemical screening and Anthelmintic activity of aqueous and ethanolic extracts of flowers of *Polyalthia longifolia (Annonaceae)*. Highest antibacterial activity was observed in ethanolic and aqueous extracts, the same was compared with the standard drug Tetracycline & Gentamycin as evident from zone of inhibition values in the concentration of 100,200,300,400 and 500 µg/ml. Phytochemical analysis of the extracts of *polyalthia longifolia* revealed the presence of steroids, alkaloids, glycosides, biterpenoids, carbohydrates, amino acids, phenolics and flavonoids as major phytochemicals. The activities observed could be attributed to the presence of some of the Phytochemical detected which have been associated with antibacterial activity. Anthelmintic activity was carried out for both the extracts on adult Indian earthworm *Pheretima Posthuma*. Both the extracts showed Anthelmintic activity at 500, 750 and 1000 µg/ml concentration. The activities were compared with the standard drug Albendazole. When the dose of the extract is increased, a gradual increase in Anthelmintic activity was observed. Ethanolic extract showed better Anthelmintic activity in comparison to the aqueous extracts of *polyalthia longifolia.* The data was verified as statistically significant by using MEAN±SEM**.**

Keywords: Antibacterial, phytochemical, *pheretima posthuma*, anthelmintic activity, *Polyalthia longifolia*.

Introduction

The use of herbal products as antimicrobial agents may provide the best alternative to the wide and injudicious use of synthetic antibiotics. The demand on plant based therapeutics is increasing in both developing and developed countries due to growing recognition that they are natural products, non narcotic, easily biodegradable producing minimum environmental hazards, having no adverse side effects and easily available at affordable prices.

Polyalthia longifolia (Annonaceae) commonly called Nettilinkam (Tamil), false ashoka, green champa, Indian mast tree, Indian fir tree, *glodogan tiang* (Indonesian) is a widely distributed tree in the Mediterranean region, west and central Asia, South Asia, South East Asia, Africa, South East America, Australia, India, China, Bangladesh and Myanmar. *Polyalthia longifolia* is a lofty evergreen tree, native to India, commonly planted due to its effectiveness in alleviating noise pollution. It is a high evergreen tree, narrow branching, about 25 - 60 ft high with long green leaves (7-15 cm in length) and round or oval shaped fruits. Fresh leaves are a coppery brown color and are soft and delicate to touch; as the leaves grow older the color becomes a light green and finally a dark green.

Helminthes infections are the most widespread infections in humans, distressing a huge population of the world. Although the majority of infections due to helminthes are generally restricted to tropical regions and cause enormous hazard to health and contribute to the prevalence of undernourishment, anaemia, eosinophilia and pneumonia. Parasitic diseases cause ruthless morbidity affecting principally population in endemic areas. The gastro-intestinal helminthes becomes resistant to currently available Anthelmintic drugs therefore there is a foremost problem in treatment of helminthes diseases. Hence there is an increasing demand towards natural Anthelmintic.

**Corresponding author*

Materials and Methods

Collection and preparation of plant samples:

The Flowers of *Polyalthia longifolia* were collected from local area of Anantapur in the month of February. The plant material was dried under shade, pulverized and stored in air tight container and used for further extraction. The plant was identified and authenticated by Dr. Prasad, Professor, department of botany, S K University, Anantapur, Andhra Pradesh.

Preparation Of Extract:

Aqueous extract (maceration method):

Powdered material of Flowers of *Polyalthia longifolia* (200gm) was kept for maceration with 1000ml of distilled water for 24hrs. The extract was double filtered by using muslin cloth and Whatman filter paper no.1 and the extract was concentrated then dried by *Heidolph* Rotavapour at 60-70^{0}C.

Alcoholic extract (maceration method):

Powdered material of Flowers of *Polyalthia longifolia* (200gm) was kept for maceration with 1000ml of ethanol for 24hrs. The extract was double filtered by using muslin cloth and Whatman filter paper no.1 and the extract was concentrated then dried by *Heidolph* Rotavapour at 60-70^{0}C.

Test of antibacterial activity [2, 3, 13]

The plate-hole diffusion assay as described by Bauer et al. (1966) and Ahmed et al. (2001) was used to determine the growth inhibition of bacteria by the plant extracts. The following bacteria obtained from Microbiology Department, JNTU-OTRI Anantapur. All bacteria were maintained at 40°C on nutrient agar plates before use. The tests were carried out by using a stock concentration of 100,200,300,400 and 500 µg/ml prepared for both standard and samples. 1 g of the aqueous and ethanolic extract was dissolved into 100 ml of distilled water and DMSO further dilutions were made by using the same solvent systems. Nutrient agar was prepared and 25 ml each was poured into sterile Petri dish. This was allowed to solidify and using a sterile cock-borer of 8 mm diameter three equidistant holes per plate were made in the set agar and were inoculated with 0.5 ml over night suspension of the bacteria. There after, the wells (holes) were filled with the extract solution at varying concentrations of 100,200,300,400 and 500µg/ml respectively. This was done in triplicate and the plates were incubated at 37°C for 24 h. The antibacterial activities were observed and measured using a transparent meter rule and recorded.

Screening of extracts for phytochemicals [1, 4, 5]

The aqueous and ethanolic extract was screened for phytochemical constituents using standard procedures of analysis (Trease & Evans; Harborne J.B).

Anthelmintic Activity [7,8,10,12,13]

Animals:

Adult earthworms (*Pheretima posthuma), were* used to evaluate Anthelmintic activity by in *vitro* method. *Earthworms* were collected near the swampy water in village marala in Anantapur district. The average size of earthworm was 8-15 cm; the same was identified by Dr. Philip, Department of Zoology, S K University, Anantapur, Andhra Pradesh.

Drugs and Chemicals:

Albendazole and Normal saline water were used during the experimental protocol. Aqueous and ethanolic extract of flowers of *polyalthia longifolia* was tested in various doses in each group. Normal saline water was used as control. Albendazole (Albendazole tablet, GSK) was used as the standard drugs for comparative study with all the extracts.

The Anthelmintic assay was carried out as per the method of Ajaiyeoba et al. The assay was performed in *vitro using* adult Indian earthworm and *Pheretima posthuma* owing to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings for preliminary evaluation Anthelmintic activity. Test samples of the extract was prepared at the concentrations, 100, 200 and 500 µg/ml in distilled water and six worms i.e. *Pheretima Posthuma* approximately equal size (same type) were placed in each nine cm Petri dish containing 25 ml of above test solution of extracts. Albendazole was used as reference standard and normal saline water as control. This procedure was adopted for earthworms. All the test solution and standard drug solution were prepared freshly before starting the experiments. Observations were made for the time taken for paralysis was noted when no movement of any sort could be observed except when the worms were shaken vigorously. Time for death of worms were recorded after ascertaining that worms neither moved when shaken vigorously nor when dipped in warm water (50^0 C).

Results

Phytochemical screening for aqueous and ethanolic extracts of flower of *Polyalthia longifolia* showed in (Table no 1). Antibacterial activity of specific concentration (100,200,300,400 & 500µg/ml) of aqueous and ethanolic extract of flowers of *Polyalthia longifolia* were documented, study revealed that both aqueous and ethanolic extract of *Polyalthia longifolia* exhibited antibacterial activity against all the reference bacterial strains. Highest antibacterial activity was observed against *K. pneumoniae* and *S.aureus* in both the extracts followed by *E. coli* in aqueous extract and *B. subtilis* in ethanolic extract. The results showed that all the bacteria are resistant to Tetracycline and Gentamycin. (Table no 2). Anthelmentic activity for the flower extracts were compared with the standard drug active against Pheretima *posthuma* showed in (Table no 3).

Table 1

Phytochemical screening of aqueous and ethanolic extracts of flowers of *Polyalthia longifolia*

Sl. No.	Extract of *P.Longifolia*	Steroid	Alkaloid	Reducing sugar	Tannins	Gums	Glycoside	Flavonoids	Saponinis	Proteins
1	*Aqueous*	_	+	+	-	-	+	+	+	+
2	*Ethanolic*	+	+	+	+	+	+	+	+	+

(+) indicate presence (-) indicate absence.

Table 2

Antibacterial activity of specific concentration (100,200,300,400 & 500µg/ml) of aqueous and ethanolic extract of flowers of *Polyalthia longifolia*

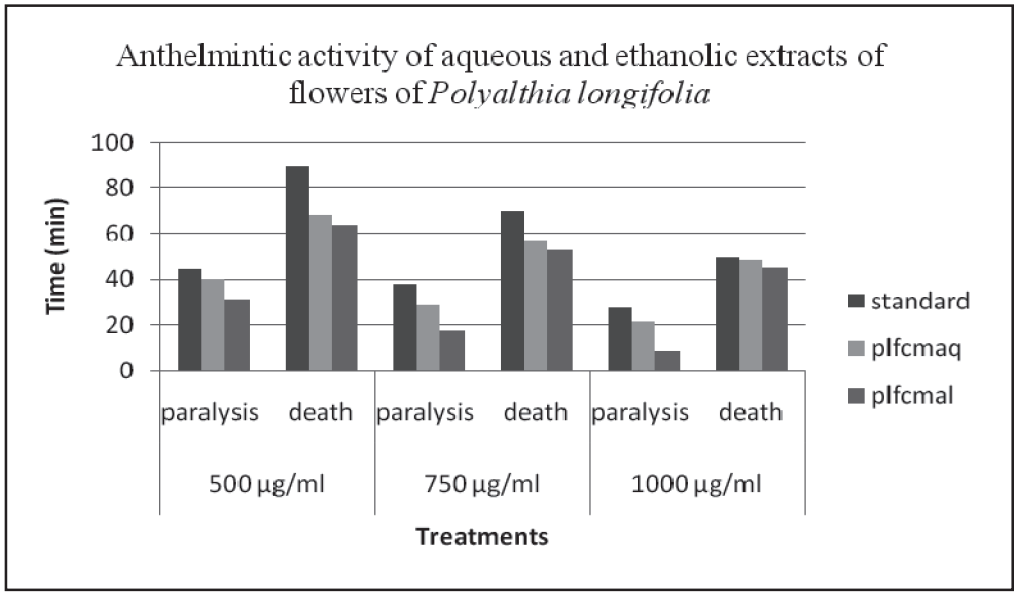
Extracts of *P. Longifolia*	Inhibition zone (mm)						
	Concentration	*B.subtilis*	*E.coli*	*P.vulgaris*	*S.aureus*	*E.aeregenes*	*K.pneumonia*
Aqueous	100µg	22±0.1	18±0.2	15±0.1	18±0.1	15±0.0	28±0.2
	200µg	20±0.2	16±0.1	16±0.2	18±0.1	16±0.1	30±0.0
	300µg	22±0.1	16±0.2	16±0.1	17±0.0	16±0.2	26±0.1
	400µg	20±0.0	14±0.1	14±0.1	17±0.0	14±0.2	26±0.1
	500µg	20±0.2	16±0.1	14±0.1	16±0.2	14±0.0	24±0.0
Ethanolic	100µg	25±0.1	14±0.1	22±0.0	27±0.1	24±0.1	27±0.1
	200µg	24±0.2	13±0.2	24±0.1	28±0.2	25±0.0	28±0.1
	300µg	22±0.1	12±0.2	24±0.1	28±0.2	27±0.1	23±0.0
	400µg	20±0.2	12±0.1	22±0.0	26±0.0	26±0.2	22±0.0
	500µg	18±0.2	11±0.1	20±0.2	27±0.1	26±0.1	24±0.1
Control	00±0.0	00±0.0	00±0.0	00±0.0	00±0.0	00±0.0	00±0.0
Tetracycline	100µg	17	12	10	00	14	11
Gentamycin	100µg	19	22	18	23	22	14

* Control set consisting of distilled water and DMSO

Table 3

Anthelmintic activity of aqueous and ethanolic extracts of flowers of *Polyalthia longifolia*

Sl. No.	Treatment	Dose(µg/ml)	Time Taken For Paralysis (Min)MEAN±SEM	Time Taken For Death (Min)MEAN±SEM
1	Control (Distilled Water)	——	——	——
2	Standard (Albendazole. Tab)	500	44.5±0.42	89.5±0.42
		750	38±0.70	70±0.70
		1000	27.3±0.49	49.3±0.47
3	Aqueous Extract of *P.Longifolia*	500	40±0.57	68±0.57
		750	29.3±0.49	57.3±0.49
		1000	21.5±0.42	48.5±0.42
4	Alcoholic Extract of *P.Longifolia*	500	31.1±0.70	64±0.70
		750	17.8±0.47	52.8±0.47
		1000	09±0.70	45±0.57



Conclusion

The Flower extracts of *Polyalthia longifolia* were screened for phytochemical, antibacterial and anthelmintic activity showed in the table no 1, 2 and 3 respectively as an indication of the possession of a wide range of pharmacological activities including anticancer, antiviral and pesticidal properties. The plants may therefore be a potential source for novel drug development. However, further studies are necessary to find out the active principles responsible for these activities.

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