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Physicochemical and Phytochemical Evaluation of Different Extracts of *Leptadenia Reticulata* (Retz.)

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ABSTRACT

Leptadenia reticulata have different indications in ayurveda and is used for several diseases. *Leptadenia reticulata* traditionally known as "jivanti", a jeevana tonic, belongs to family Asclepidaceae. The present study comprises physico-chemical and phytochemical evaluation of different extracts of *Leptadenia reticulata* by using standard methods. The physical evaluation was carried out for the determination of extractive values, ash values and moisture content. Whereas phytochemical evaluation was carried out for the presence of carbohydrates, glycosides, flavonoids, tannins, phytosterols and phenolic compounds in different extracts of *Leptadenia reticulata.* Results revealed the presence of carbohydrates, flavonoids, tannins, phytosterols and phenolic compounds. This study will help in determining the quality and purity of a crude drug and laying down pharmacopoieal standards for *Leptadenia reticulata.*

Key words: Jivanti, *Leptadenia reticulata,* Flavonoids, Phytochemical Evaluation.

Introduction

Leptadenia reticulata (Retz.) Wight and Arn Synonym: Cynanchum reticulatum (Retz.) belonging to family Asclepidaceae is commonly known as Dodi (Hindi, Guj. & Mar.), Jivanti (Sansk.), Palaikkodi (Tamil) [1-5]. It is twining shrub, distributed in the southern parts of India and grows in sub-himalayan tracts of Punjab, Gujarat, Uttar Pradesh and throughout peninsular India, ascending upto an altitude of 900m.

The whole plant ameliorates 'tridoshas' (Vatta, Pitta and Kapha), and is of great value in general debility, involuntary seminal discharge, as a stimulant and snake bite[6-7], abortifacient, tonic, restorative, bactericidal, antifebrifuge, wound healer and in mouth ulcer [8]. Roots are used in many ayurvedic and herbal formulations [9] in diseases of the ear and nose, skin infections and general debility [10]. The major therapeutic claim is its galactogogue action, which has been proved in rats, cows [11-12] as well as humans [13] and to increase the egg laying capacity of hen in poultry industry. Flowers are good for eyesight. It nourishes and rejuvenates the body and increases memory, longevity, immunomodulation and adoption. The leaves are useful in asthma and cough and against ringworms [14]. Aqueous extract of the stem demonstrated negative, chronotropic and prolonged hypotensive effect in dogs [15]. Aqueous extracts are safely tolerable up to a dose of 3.125g/kg [16].

Previously reported chemical constituents of *Leptadenia reticulata* are á-amyrin, â-amyrin, ferulic acid, luteolin, diosmetin, rutin, â-sitosterol, stigmasterol, hentriacontanol [17], a triterpene alcohol simiarenol [18] and api-genin [19]. Pregnane glycosides reticulatin, deniculatin and leptaculatin have also been isolated from the aerial parts [20] which on hydrolysis give calogenin, tocopherols. The present investigations include determination of physiochemical constants and the phytochemical screening of the different extracts of *Leptadenia reticulata* (Retz.) Wight. & Arn.

Materials and Methods

Collection of plant material

The plant material of *Leptadenia reticulata (Retz.)*

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was collected from Tirumala hills after taxonomic verification and were identified and authenticated in Department of Botany, S.V.University, Tirupati. The plant materials were coarsely powdered using a rotary grinder and stored in airtight plastic containers. This powder was used for all phytochemical analysis.

Preparation of extracts

The freshly collected plant material was washed, dried at room temperature for 15-20 days under shade and was treated with a rotary grinder for size reduction. The fine powder was collected and was used for preparation of extracts. Dried plant material (100 g) was extracted with Soxhlet apparatus using 400 mL petroleum ether for about 48h. After defatting, the marc was dried in hot air oven at 50°C, packed in Soxhlet apparatus and further extracted with 400 mL 95% ethanol until it does not show the presence of any residue on evaporation. The aqueous extract was prepared by cold maceration with 3% methanol-water for 7 days with occasional shaking. The solvents were removed from the extracts under reduced pressure by using rotary vacuum evaporator.

Physicochemical evaluations

Moisture content

An accurately weighed quantity of the shade dried powder of *Leptadenia reticulata* (3 g) was taken in a tared glass bottle and the initial weight was taken. The crude drug was heated at 105°C in an oven and weighed. This procedure was repeated till a constant weight was obtained. The moisture content of the sample was calculated in the percentage with reference to shade dried plant powder using formula [21].

$$\% \text{ Moisture content} = \frac{\text{loss in weight of the sample}}{\text{Weight of the sample}} \times 100$$

Ash values [22]

Determination of total ash

An accurately weighed quantity of the shade dried powder of *Leptadenia reticulata* (2 g) was incinerated in a crucible at a temperature of 450ÚC in a muffle furnace till carbon free ash was obtained. It was then cooled and weighed. The percentage of total ash was calculated with reference to the shade dried plant powder using following equation.

$$\% \text{ Total ash value} = \frac{\text{Weight of total ash}}{\text{Weight of the crude drug taken}} \times 100$$

Determination of acid insoluble ash

Ash obtained was boiled for 5min with 25 mL of 2 M HCl and filtered using an ash less filter paper. Insoluble matter retain on filter paper was washed with hot water and filter paper was burnt to a constant weight in a muffle furnace. The percentage of acid insoluble ash was calculated with reference to the shade dried plant powder using following equation.

$$\% \text{ acid insoluble ash value} = \frac{\text{Weight of acid insoluble ash}}{\text{Weight of the crude drug taken}} \times 100$$

Determination of water soluble ash

Ash above obtained, was boiled for 5min with 25 mL of distilled water, cooled and the insoluble matter was collected on an ash less filter paper. Paper was washed with hot water and ignited for 15min at a temperature not exceeding 450ÚC in a muffle furnace. Difference in weight of ash and weight of water insoluble matter gave the weight of water soluble ash. The percentage of water soluble ash was calculated with reference to the shade dried plant powder using formula.

$$\% \text{ Water soluble ash value} = \frac{\text{Weight of total ash- Weight of water insoluble ash}}{\text{Weight of the crude drug taken}} \times 100$$

Extractive values [23]

Extractive values of shade-dried powder of *Leptadenia reticulata* were determined using following methods.

a) Determination of alcohol soluble extractive

An accurately weighed quantity of the shade dried powder of *Leptadenia reticulata* (5 g) was macerated with 100 mL of alcohol (Ethanol) in a closed flask for 24 h, shaking frequently during the first 6 h. It was then allowed to stand for 18 h and filtered rapidly to prevent any loss during evaporation. Evaporate 25 ml of the filtrate to dryness in a porcelain dish and dried at 105ÚC and weighed. The percentage of alcohol (Ethanol) soluble extractive was calculated with reference to the shade dried plant powder.

b) Determination of water soluble extractive

Weighed quantity of the shade dried powder of *Leptadenia reticulata* (5 g) was macerated with 100 mL of water in a closed flask, shaking frequently during the first 6 hrs and allowed to stand for 18 hrs. Thereafter it was filtered taking precaution against loss of water. Evaporate 25 mL of filtrate to dryness in a tared flat bottom shallow dish dried at 105°C and weighed. The percentage of water soluble extractive was calculated with reference to the shade dried plant powder.

c) Determination of petroleum ether soluble extractive

Weighed quantity of the shade dried powder of *Leptadenia reticulata* (5 g) was macerated with 100 mL petroleum ether in a closed flask for 24 h, shaking frequently during the first 6 hrs and allowed to stand for 18 hrs. Thereafter it was filtered rapidly taking precaution against

loss of petroleum ether due to its volatility. Evaporate 25 mL of filtrate to dryness in a porcelain dish and dried at 105°C and weighed. The percentage of petroleum ether soluble extractive was calculated with reference to the shade dried plant powder.

Phytochemical Evaluation

The freshly prepared petroleum ether, methanolic and aqueous extracts of *Leptadenia reticulata* were qualitatively analyzed for the presence of major phytochemical constituents using the standard procedures.

1. Detection of Carbohydrates [24]

100 mg of extracts were dissolved in 10 mL of water and filtered. The filtrate was used to test the presence of proteins and amino acids.

(a) Molisch's Test

To the 1mL of filtrate, 2 drops of Molisch's reagent was added in a test tube and 2 mL of concentrated sulphuric acid were added carefully down the side of the test tube. Formation of violet color at the interface indicates the presence of carbohydrates.

(b) Fehling's Test

To the 1 mL of filtrate, 4 mL of Fehling's reagent (2 ml Fehling A and 2 ml Fehling B solutions) was added in a test tube and heated for 10 minutes in a water bath. Formation of red precipitate indicates the presence of reducing sugar.

(c) Barfoed's Test

1 mL of Barfoed's reagent is heated with 5 drops of filtrate in a test tube on boiling water bath. Formation of a brick-red precipitate within five minutes indicates the presence of monosaccharides. Disaccharides generally don't give any reaction even for ten minutes

2. Detection of Proteins and Amino acid [25]

100 mg of extracts were dissolved in 10 mL of water and filtered. The filtrate was used to test the presence of proteins and amino acids.

(a) Millon's Test

Two mL of filtrate was treated with 2 mL of Millon's reagent in a test tube and heated in a water bath for 5 minutes, cooled and few drops of $NaNO_2$ solution was added. Formation of white precipitate, which turns to red upon heating, indicates the presence of proteins and amino acids.

(b) Ninhydrin Test

To the 2 mL of filtrate, 2-3 drops of Ninhydrin reagent was added in a test tube and boiled for 2 minutes. Formation of distinct blue colour indicates the presence of amino acids.

(c) Biuret Test

2 mL of filtrate was treated with 2 mL of 10% sodium hydroxide solution in a test tube and heated for 10 minutes, a drop of 7% of copper sulphate was added in the above solution. Appearance of violet colour confirms the presence of proteins.

3. Detection of Glycosides [26]

0.5 g of extract was hydrolyzed with 20 mL of dilute hydrochloric acid (0.1N) and filtered. The filtrate was used to test the presence of glycosides.

(a) Legal Test

One mL of filtrate, 3 mL of sodium nitropruside in pyridine and methanolic alkali (KOH) was added in a test tube. A blue colour in the alkaline layer indicates the presence of glycosides.

(b) Keller-killiani Test

One mL of filtrate was shaken with 1 mL of glacial acetic acid containing traces of ferric chloride. One mL of concentrated sulphuric acid was added carefully by the side of test tubes. A blue colour in acetic acid layer and red colour at the junction of the two liquids indicates the presence of glycosides

(c) Modified Borntrager Test

One mL of filtrate, 2 mL of 1% ferric chloride solution was added in a test tube and heated for 10 minutes in boiling water bath. The mixture was cooled and shaken with equal volume of benzene. The benzene layer was separated and treated with ammonia solution. Formation of pink colour in the ammonical layer indicates the presence of glycosides.

4. Detection of Alkaloids [27]

0.5 g. of extract was dissolved in 10 mL of dilute hydrochloric acid (0.1N) and filtered. The filtrate was used to test the presence of alkaloids.

(a) Dragendorff's Test

To the 2 mL of filtrate add 2-3 drops of Dragendorff's reagent. Formation of reddish brown colored precipitate indicates the presence of alkaloids.

(b) Hager's Test

To the 2 mL of filtrate add Hager's reagent. Formation of yellow colored precipitate indicates the presence of alkaloids.

(c) Mayer's Test

To the 2 mL of filtrate add 2-3 drops of Mayer's reagent, formation of cream colored precipitate indicates the presence of alkaloids.

(d) Wagner's Test

To the 1 mL of the extract, add 2 mL of Wagner's reagent, formation of reddish brown precipitate indicates the presence of alkaloids.

5. Detection of Flavonoids [28]

(a) Shinoda Test

To the 100 mg of extract, few fragments of magnesium metal was added in a test tube, followed by drop wise addition (3 to 4 drops) of concentrated hydrochloric acid. Formation of magenta colour or light pinkcolour indicates the presence of flavonoids.

(b) Alkaline Reagent Test

To the 100 mg of extract, few drops of sodium hydroxide solution was added in a test tube. Formation of intense yellow colour that becomes colourless on addition of few drops of dilute hydrochloric acid indicates the presence of flavonoids.

(c) Fluroscence test

To the 100 mg of extract add 0.3 mL boric acid solution (3 %w/v) and 1 mL oxalic acid solution (10 %w/v) and evaporated to dryness. The residue was dissolved in 10 mL ether; ethereal layer shows greenish fluorescence under UV light indicates presence of flavanoids.

6. Detection of Phenolic Compounds and Tannins [29]

100 mg of extract was boiled with 1 mL of distilled water and filtered. The filtrate was used for the following test.

(a) Ferric Chloride Test

To the 2 mL of filtrate in a test tube add 2 mL of 1% ferric chloride solution. Formation of bluish to black colour indicates the presence of phenolic nucleus.

(b) Lead Acetate Test

To the 2 mL of filtrate, few drops of lead acetate solution was added in a test tube. Formation of yellowish precipitate indicates the presence of tannins.

7. Detection of Fats and Oils [30]

Oily Spot Test

One drop of extract was placed on filter paper and solvent was allowed to evaporate. An oily stain on filter paper indicates the presence of fixed oil.

8. Detection of Saponins [31]

Foam Test

Dilute 1 mL of extract with 20 mL distilled water and shaken in a graduated cylinder for 15 minutes. 1 cm layer of foam indicates the presence of saponins.

9. Detection of Phytosterols [32]

0.5 g of extract was treated with 10 mL of chloroform and filtered. The filtrate was used to test the presence of phytosterols and triterpenoids.

(a) Libermann's Test

To the 2 mL of filtrate in hot alcohol, few drops of acetic anhydride was added. Formation of brown precipitate indicates the presence of sterols.

(b) Salkowski Test

To the extract solution, few drops of concentrate sulfuric acid was added, shaken and then allowed to stand, lower layer turns red indicating the presence of sterols.

Statistical Analysis

The values are represented as mean ± S.D. (n=3) and results were analyzed using ANOVA, followed by Dunnett's test where $P<0.05$ was considered statistically significant.

Results and Discussion

Leptadenia reticulata was subjected to systematic physicochemical and phytochemical screening by successively extracting with various organic solvents of increasing polarity to determine the amount of soluble constituents in a given amount of plant material. The data generated is helpful in determining the quality and purity of a crude drug, especially in the powdered form. In this study the parameters used for the evaluation of *Leptadenia reticulata* were moisture content, extractive values by different solvents (includes petroleum ether, methanol and water), ash values (total ash, water soluble and acid insoluble ash) (Table 1). The objective of reducing the vegetable drug to its ash is to remove all traces of organic matter, which may otherwise interfere in an analytical determination. On incineration, crude drugs leave an ash usually consisting of carbonates, phosphates and silicates of sodium, potassium, calcium and magnesium. The determination of ash is useful for detecting low-grade products, exhausted drugs and excess of sandy or earthy matter which is more especially applicable to powdered drugs.

Phytochemical analysis was performed on the petroleum ether, methanol and aqueous extracts of *Leptadenia reticulata*. Petroleum ether extract was found to contain phytosterols, fats and oils. Methanolic extract contains flavonoids, phenolic compounds, alkaloids and glycosides. Aqueous extract contains glycosides, proteins and amino acids, carbohydrates, phenolic compounds, tannins and saponins (Table 2).

Conclusion

In this project, it was planned to present work on Ayurvedic plant drug as an effort to highlight the potential of these plants as a valuable resource of biologically active molecules. Herbal remedies have been common place in various cultures throughout recorded history, and still serve as the main means of therapeutic medical treatment. The recent years have witnessed a resurgence of interest in natural medicines world over as people are turning to use medicinal plants and phyto-chemicals in health care. India has one of the oldest cultural traditions of use of its medicinal flora since Vedic period. Ayurveda, Unani, Siddha and other traditional systems of medicine are one of the oldest systems and utilize large number of medicinal plants. In contrast, other approaches such as phytochemical screening massive biological screening of randomly collected plants and phytochemical examination of plants

Table 1:

Physico chemical evaluation of whole plant of *Leptadenia reticulata*

S.No	Quality Parameters	Results
1.	Moisture content	6.7 ± 0.132
2.	Ash value	
	A. Total ash value	7.2 ± 0.243
	B. Acid insoluble ash	0.9 ± 0.221
	C. Water soluble ash	2.7 ± 0.123
3.	Extractive value	
	A. Alcohol soluble extract	6.4 ± 0.324
	B. Water soluble extract	8.5 ± 0.453
	C. Petroleum ether soluble extractive	6.2 ± 0.231

Mean ± SD, Number of readings (n)=3

Table 2:

Phytochemical analysis of different extracts of whole plant of *leptadenia reticulata*

S.No	Tests	Petroleum ether extract	Methanolic extract	Aqueous extract
1	Carbohydrates	–	–	+
2	Proteins and aminoacids	–	–	+
3	Glycosides	–	+	+
4	Alkaloids	–	+	–
5	Flavonoids	–	+	-
6	Phenolic compounds	–	+	+
7	Tannins	–	–	+
8	Saponins	–	–	+
9	Phytosterols	+	–	–
10	Fats and oils	+	–	–

with the aim of identifying new chemical compounds have proved to be very helpful in discovering new drugs.

Leptadenia reticulata, traditionally known as *Jivanti,* is very important medicinal plant belonging to family Asclepidaceae. The present study concluded that the plant *Leptadenia reticulata* contains variety of phytoconstituents. The physicochemical evaluation revealed that the standard quality and purity of drug. The objective of ashing vegetable drugs is to remove all traces of organic matter, which may otherwise interfere in an analytical determination. On incineration, crude drugs normally leave an ash usually consisting of carbonates, phosphates and silicates of sodium, potassium, calcium and magnesium. Phytochemical studies on the extracts of *Leptadenia reticulata* (stems) showed presence of phytosterols, carbohydrates, glycosides, flavonoids, tannins & phenolic compounds. This information may be further used for isolation of various compounds from *Jivanti* for treatment of diseases for human beings.

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A New Analytical Reagent 2, 6-Diacetylpyridine Bis(4-phenyl- 3-thiosemicarbazone) for the Determination of Ni(II) in Medicinal leaves and Soil Samples

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ABSTRACT

A new synthesized reagent 2, 6-diacetylpyridine bis(4-phenyl-3-thiosemicarbazone) [2, 6-DAPBPTSC] is proposed as a sensitive and selective analytical reagent for the extractive spectrophotometric determination of Nickel(II). It forms a yellow colored complex with 2, 6-DAPBPTSC at pH 4.0 which was extracted into isoamylalcohol, under optimum conditions. The maximum absorption of the complex extracted was measured at 400 nm. The Beers law was obeyed in the range of 0.58 to 5.87 ppm. The molar absorptivity and Sandells sensitivity of complex were calculated as 1.44 $x10^4$ L mol^{-1} cm^{-1} and 4.076 $x10^{-3}$ µgcm^{-2}, respectively. The instability constant of complex was calculated from Asmus method is $6.441x10^{-5}$. The precision and accuracy of method was checked with calculation of relative standard deviation (n= 5) was 0.256 and the detection limit value was 0.0053 µgml^{-1}. The method was successfully employed for the determination of Ni (II) in medicinal leaves and soil samples. The validity of the method was tested by comparing the results with those obtained using an atomic absorption spectrophotometer.

Keywords: Nickel(II), 2, 6-diacetylpyridine bis(4-phenyl-3-thiosemicarbazone) [2,6-DAPBPTSC], medicinal leaves and soil samples.

Introduction

Nickel is an element, which has high industrial importance, due to its high strength and resistance to corrosion in many media. Nickel is used in ceramic industry, and also used in batteries. An important use of nickel in food industry is as a catalyst in the hydrogenation of oils. It used in Nickel plating and also in the manufacture of alloys Nickel can be released into the atmosphere from several industrial processes, including oil and coal burning power plants, trash incinerators, and metallurgy industry. Moreover, Nickel can enter into aquatic body from dissolution of oxide and sulphide rocks containing nickel combined with other elements. The excellent chromophoric properties of nickel (II) enable it by giving colorured products with many organic reagents.. The high concentration of the nickel does show serious health hazards. The high incident of respiratory track moplacia and dermatitis has been observed when exposed by nickel refineries. In views of this separation and determination of nickel from associated elements is indispensable. Thus, the exposition of nickel may take place by air, food samples, drinking water and mainly by skin contact with contaminated soils and alloys containing nickel[1]. Another common disease caused by skin disorder is known as nickel-eczema. This disease is more pronounced in workers daily exposed to great amounts of nickel and in those people who are sensitive to nickel[2].

For the determination of nickel in trace amount levels, there are several frequently adopted methods using analytical techniques, such as AAS, ICP-AES, ICP-MS, X-ray fluorescence spectroscopy, spectrophotometry, spectrofluorometry and so on. Among these, the spectrophotometric method is preferred because it is cheaper and easy to handle, with comparable sensitivity and accuracy, besides having good precision.

There are many organic complexing reagents [3-7], which can be used for the spectrophoto-metric determination

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of nickel, yet these suffer from disadvantages such as low sensitivity, incomplete extraction and interference from a large number of foreign ions (Table 1).

A survey of literature reveals that only a few phenylthiosemicarbazones are employed for extractive spectrophotometric determination of Ni(II). Hence, the authors have introduced a new sensitive reagent 2,6-diacetylpyridine bis(4-phenyl-3-thiosemicarbazone) (2,6-DAPBPTSC) for the extractive determination of trace amounts of Ni(II). The proposed method, when compared with other spectrophotometric methods, is more sensitive and selective. It also offers advantages like reliability and reproducibility in addition to its simplicity, instant color development and less interference. The results obtained through UV–Vis spectrophotometer have been compared with those obtained through the atomic absorption spectrometer.

Apparatus

A Shimadzu (UV-2450 model) UV–vis spectrophotometer with 1.0 cm quartz cell has been used for absorbance measurements. An Elico (model Li-10 India) pH-meter has been used for pH adjustments. A Hitachi model 170-30 atomic absorption spectrophotometer was used for comparison of results.

Reagents

All of the chemicals used are of analytical grade; quartz processed high-purity water has been used throughout the experiment.

Synthesis of 2,6-diacetylpyridine bis(4-phenyl-3-thiosemicarbazone) (2,6-DAPBPTSC)

2,6-diacetylpyridine bis-4-phenyl-3-thiosemicarbazone (2,6-DAPBPTSC) is prepared as per the procedure: 1.60 g of 2,6-diacetylpyridine is dissolved in 30 mL of absolute ethanol and mixed in a round-bottomed flask with 2.20 g of 4-phenyl-3-thiosemicarbazide dissolved in 30 mL ethanol. The mixture was heated under reflux for 3 h and then allowed to cool to room temperature and kept for 12 h. The crystals obtained are filtered and washed with cold ethanol and then recrystalized from ethanol. The melting point was 152°C. 2,6-DAPBPTSC dissolves in N, N-dimethylformamide (DMF), acetone and dimethyl sulphoxide. The characterization of 2,6-DAPBPTSC was carried out by IR and ^{1}H NMR spectroscopy. The IR spectrum of 2,6- DAPBPTSC shows absorption bands around 1,485 cme1(C=S), 1,540 cme1 (C=N), and 3,300 cme1 (eNH). The 1H NMR (DMSO, ppm) 9.85 (eNH), 2.6 (eCH3), and 7.2–7.8 (pH).

Preparation of standard solution of nickel (II).

A total of 6.73 g of ammonium nickel sulfate hexahydrate [$(NH_4)_2Ni(SO_4)_26H_2O$)] was weighed, dissolved in double-distilled water containing a few drops of concentrated sulfuric acid and made up to 1L. The Stock solution was then standardized gravimetrically using dimethylglyoxime (Vogel, 1961). The required dilute solution of nickel (II) was prepared by diluting the stock solution with double-distilled water. All reagents used were of analytical reagent grade unless otherwise stated.

Buffer solutions

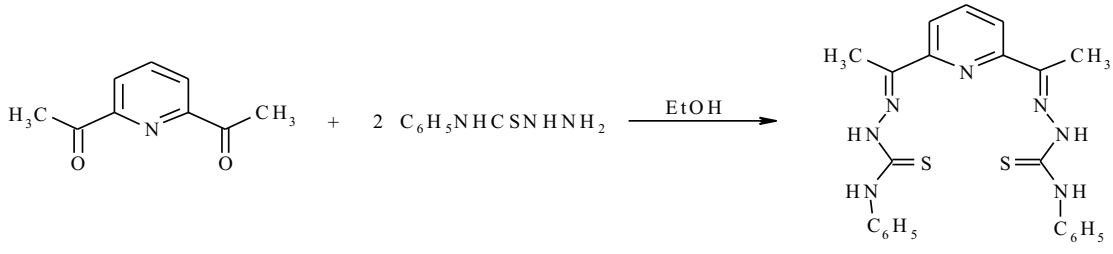
A total of 1.0 mol L^{-1} sodium acetate and 1.0 mol L^{-1} acetic acid solutions were prepared in double-distilled water. Suitable portions of these solutions were mixed to get the desired pH.

Collection of medicinal leaves, soil samples preparation of solutions and analytical procedure

The environmental matrices (soil and medicinal leaves) to be analyzed were collected in and around Tirupati, Andhra Pradesh, South India. The dried sample was pulverized in a mortar for the purpose of analysis, to a convenient size. An aliquot of 500 mg of soil/100 mL of medicinal leaves was digested with 5mL of HNO_3 (65%) in a teflon vessel.

Analytical procedure for standard alloy samples

A total of 0.1 g of each oven-dried (110 ^{0}C) alloy sample was dissolved in 15 mL of aqua regia. The solution was heated to near dryness and nitrate was expelled from the residue, using 5 mL of concentrated hydrochloric acid. Each residue was extracted into double-distilled water, separately, and made up to 100 mL. The sample was digested for 3 h at 80 ^{0}C, and again digested at 160 ^{0}C for 45 min. After treating with double-distilled water the supernatant liquid was made up to the mark in a 25 mLstandard flask.



2,6-diacetylpyridine **4-phenylthiosemicarbazide** **2,6-diacetylpyridine bis-4-phenylthiosemicarbazone**

General Procedure

To an aliquot of a working standard solution containing 1.0×10^{-4} to 15.0×10^{-4} mol L^{-1} nickel(II) is added pH 4.0 buffer (2.0 mL), reagent solution (1.0 mL of 1.5×10^{-3} mol L^{-1}) and a salting-out agent 0.1 mol L^{-1} magnesium sulfate (1.0 mL). The mixture is shaken with isoamylalcohol (10.0 mL) portions twice each time for 2 min and allowed to stand for a few minutes. The organic phases are collected and made up to 25 mL with isoamylalcohol, while its absorbance is measured at 400 nm, against the reagent blank.

Results and Discussion

2,6-diacetylpyridine-bis-4-phenyl-3-thiosemicarbazone (2,6-DAPBPTSC) forms a 1:1 (M/L) yellow colored complex with nickel(II), which is extracted into isoamylalcohol, from sodium acetate -acetic acid (pH 4.0) buffer. The yellow colored Ni(II)–2,6-DAPBPTSC complex has a maximum absorbance at 400 nm and is stable for 48 h. The conditions for effective extraction are established after studying the influence of various factors, such as pH, choice of the solvent, reagent concentration, salting-out agent, and influence of diverse ions, in order to develop a selective and sensitive extractive spectrophotometric method for the determination of nickel(II) in trace level.

Absorption spectra of the reagent and Ni (II)–2,6-DAPBPTSC complex

The absorption spectrum of Ni(II)-2,6-DAPBPTSC complex was recorded against the reagent blank. Similarly the absorption spectrum of the reagent (2,6-DAPBPTSC) was recorded against the solvent blank. The absorption spectra of both the complex and reagent are shown in Fig. 1. The obtained spectra reveal that the Ni(II)-2,6-DAPBPTSC complex and the reagent have shown maximum absorbance at 400 nm and 360 nm, respectively. The reagent has minimum absorbance at the maximum absorbance of the complex and hence the reagent does not interfere with the determination of nickel(II). Therefore, further all the spectral measurements of the complex have been carried out at 400 nm.

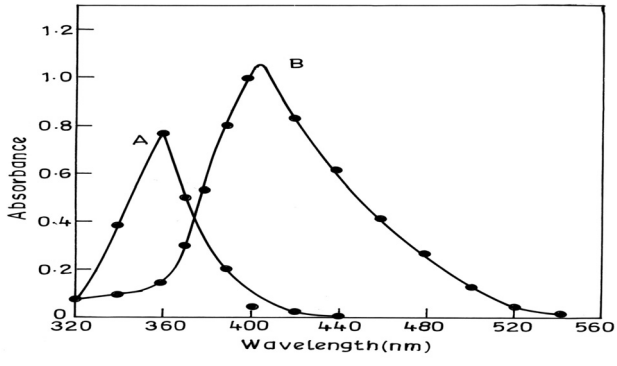


Fig.1 : A. Absorption spectrum of 2,6-DAPBPTSC Vs isoamyl alcohol blank.
B. Absorption spectrum of Ni(II)-2,6-DAPBTSC complex Vs 2,6-DAPBPTSC blank

Effect of pH on the extraction of Ni(II)–2,6-DAPBPTSC complex

To arrive the optimum pH required for maximum color development, the influence of pH on the color intensity was studied by using differs in pH range from 1.0-6.5 (Fig. 2). The absorbance of the Ni(II)-2,6-DAPBPTSC complex increases as the pH increases from 1.0-3.0 and remains constant in the pH range 3.5-4.5. However, it has decreased beyond 4.5. Hence, pH 4.0 is chosen for further studies, considering this as an optimum pH.

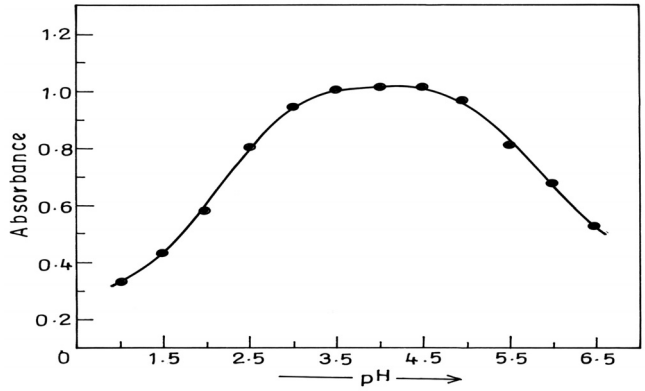


Fig.2 : Effect of PH on the extraction of Ni(II)- 2,6-DAPBPTSC Complex

Effect of reagent concentration on the absorbance of Ni(II)-2,6-DAPBPTSC complex

The effect of reagent concentration on the formation of the Ni(II)-2,6-DAPBPTSC complex has been studied by keeping the constant concentration of metal ion solution (1.0×10^{-4} mol L^{-1}) with different reagent solution concentration ranging from 1.0×10^{-4} to 15.0×10^{-4} mol L^{-1} in order to get the maximum color formation. The colored solutions were collected into 25.0 mL standard flasks and made up to the mark isoamylalcohol. The absorbances of the organic phases were measured at 400 nm against their corresponding reagent blank. It is clearly observed from the absorbance values that a maximum thirteen-fold molar excess of the reagent is sufficient to get a maximum color formation of the complex.

Effect of solvents on the extraction of Ni(II)–2,6-DAPBPTSC complex

The extraction of Ni(II)-2,6-DAPBPTSC complex into various organic solvents are studied, such as chlorobenzene, benzene, carbon tetrachloride, n-butanol, methylisobutyl ketone, cyclohexanol, n-amylalcohol, isoamylalcohol, chloroform, cyclohexane and 1-propyl acetate. Among all the solvents tested, isoamylalcohol was found to be the most effective extracting complex. Hence, isoamylalcohol is chosen as a suitable solvent for further investigations.

Effect of salting-out agents on the extraction of Ni(II)-2,6-DAPBPTSC complex

Various salting-out agents, such as magnesium sulfate, magnesium nitrate, lithium acetate, lithium sulfate, lithium

nitrate and ammonium sulfate were employed to enhance the metal complex extraction into organic solvent in a single step. After the studies, it was observed that the presence of 0.1 mol/l magnesium nitrate solution enhanced the extraction. The presence of nickel (II) in the aqueous phase after extraction was tested gravimetrically by using dimethylglyoxime. It was found that with magnesium nitrate as a salting-out agent, the complex was extracted quantitatively into isoamylalcohol.

Validity of Beer's law, molar absorptivity, Sandell's sensitivity and correlation coefficient for Ni(II)-2,6-DAPBPTSC complex

The Ni(II)-2,6-DAPBPTSC complex followed Beer's law in the range of 0.587 to 5.87 ppm (Fig. 3). The molar absorptivity of the complex was calculated to be 1.44×10^4 L mol^{-1} cm^{-1} and Sandell's sensitivity of complex obtained from Beer's law data for absorbance 0.001, was found to be 0.0040 µg cm^{-2}. The correlation coefficient value of the Ni(II)-2,6-DAPBPTSC complex, with an independent variable as concentration in µg mL^{-1} and dependable variable as absorbance, was found to be 0.972. This indicates the linearity between the two variables.

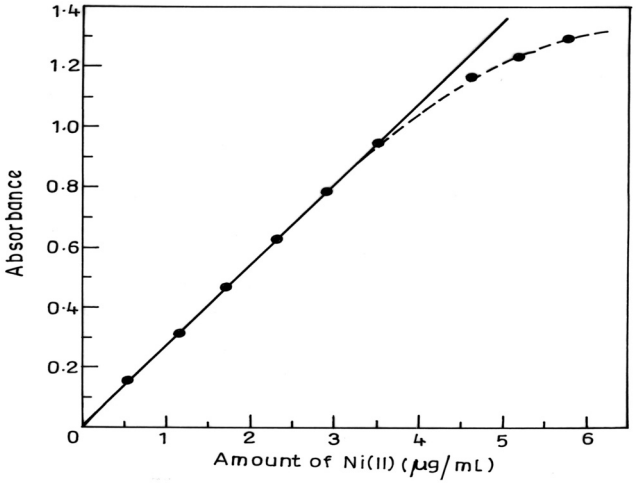


Fig. 3: Applicability of Beer's Law for Ni(II)-2,6-DAPBPTSC Complex

Ringbom plot for Ni(II)–2,6-DAPBPTSC complex

Ringbom's plot is the standard adopted to know the optimum range of the concentration for a system, which obeys Beer's law. A Ringbom's plot was drawn between logC of nickel(II) and (1-T), where 'T' is the transmittance, shown Fig. 4. The plot has a sigmoid shape with linearity at intermediate concentration values of from 2.95 to 3.65 ppm, which indicates that nickel(II) is precisely determined in the optimum concentration range 2.95 to 3.65 ppm. The slope of the Ringbom's plot is 0.74. Based on this value, the ratio between the relative error in concentration and photometric error is 3.112. For a photometric error of one percent, ΔP=0.001. Hence, the relative error in concentration is 0.03112.

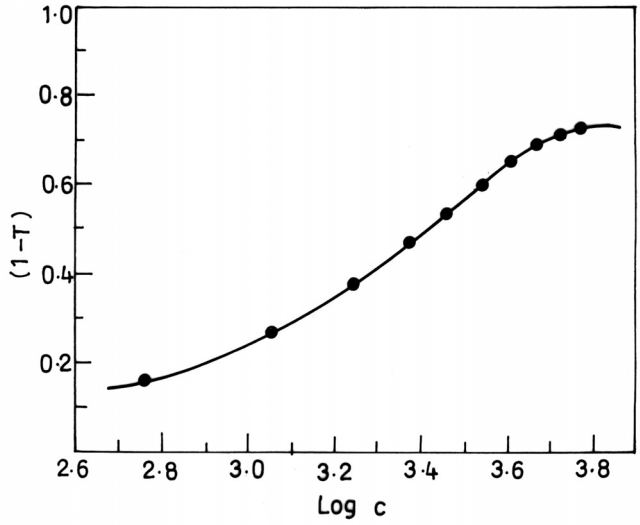


Fig. 4 : Ringbom's plot for Ni(II)-2,6-DAPBPTSC Complex

Precision and accuracy

To assess the precision and accuracy of the method, estimations were carried out for a set of five determinations, with different concentrations of nickel(II), under optimum conditions. The obtained results reveal that the standard deviation of method was not more than 0.00158 and relative standard deviation was less than 0.256%. It is evident from these results that this method is precise, besides being accurate. The detection limit was determined as the amount of nickel(II) corresponding to thrice the standard deviation blank value, 0.00536 µg mL^{-1} was obtained.

Determination of the composition of Ni(II)-2,6-DAPBPTSCcomplex

The composition of the Ni(II)-2,6-DAPBPTSC complex was determined by the job's method. The absorbance of Ni(II)-2,6-DAPBPTSC complex was measured at equimolar solutions of Ni(II) and 2,6-DAPBPTSC were used to determine the metal to ligand ratio by job's method of continuous variation. The plot (Fig. 5) was drawn between the absorbance and V_M/V_M+V_L, where V_L and V_M are the volumes of the reagent and metal, respectively. The obtained curve indicates that the metal to ligand ratio of the extracted complex is 1:1. This was verified by slope ratio, molar ratio and Asmus'methods. The results were discovered to be in good agreement with the job's method.

Molar Ratio Method

Different aliquots of mixtures containing 1.0 ml of 1.0×10^{-3} M nickel (II) and 2.0 ml of Sodium Acetate – Acetic acid Buffer (pH 4.0) are prepared. To each of these solutions varying concentration of the 2,6-DAPBPTSC solution (0.2×10^{-3} M – 2.0×10^{-3} M) were added and final volumes made upto 10 ml with distilled water. Each of these mixtures were shaken with 10.0 ml of isoamylalcohol for 2 minutes and allowed to settle. The organic phases

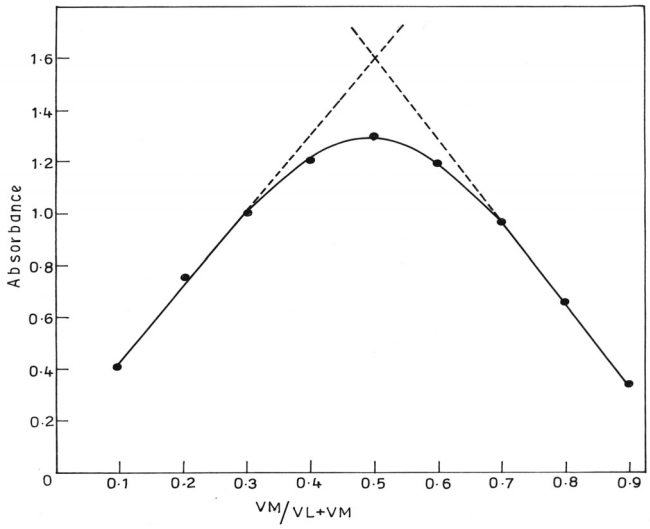
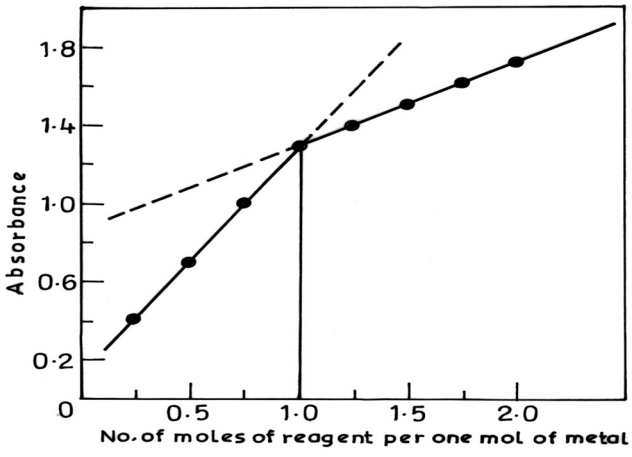


Fig.5 : Job's method of continuous variation Ni(II)-2,6-DAPBPTSC Complex

were collected in different 25 ml standard flasks and made upto the mark with isoamylalcohol. The absorbences of the organic phases obtained were recorded at 400 nm, against their corresponding reagent blanks. A plot drawn between the absorbance and number of moles of the ligand per mole of the metal ion is shown in Fig. 6.



Determination of instability constant Ni(II)-2, 6-DAPBPTSC complex

The instability constant of Ni(II)-2,6-DAPBPTSC complex was calculated using Asmus'method. The absorbance values of the extract were obtained at 400 nm after shaking the solutions containing fixed volumes of 1.0 × 10^{-3} mol L^{-1} nickel(II), buffer (pH, 4.0) and different known volumes (0.25-2.02 mL of 1.0 × 10^{-3} mol L^{-1}) of the 2,6-DAPBPTSC solution with isoamylalcohol. The instability constant of nickel(II)-2,6-DAPBPTSC solution was calculated and found to be 1.44 × 10^{-4}, at room temperature by Asmus'method.

Effect of foreign ions on the extraction of Ni (II)-2, 6-DAPBPTSC complex

The tolerance limit means that the limit which the foreign ion cannot be interfere for the determination of Ni(II). In order to assess the possible analytical applications of this developed method, the effect of some foreign ions are also examined, by carrying out determinations of 75.0 μg of nickel(II) with a known amount of the ion in question, using the recommended general procedure. The criterion for interference is a variation of more than ± 2 percent in absorbance from the expected value for nickel(II) alone (Table 1). In respect of some interfering ions, an increased tolerance limit is achieved by the addition of masking agents, such as thiosulfate, fluoride, tartrate or thiocyanate. Increasing the amount of masking agents proportionately could mask higher amount of interfering ions. In this study, cations like As(III), As(V), Mg(II), Mn(II), Zr(IV), Sb(III), Ca(II), Sr(II), Ba(II), and Tl(III) do not interfere, when present up to 5500 μg and cations like Bi(III), Hg(II), Be(II), Th(VI), U(VI), Al(III), and V(V) are tolerated up to 3000 μg, but Co(II), Cu(II), Zn(II), Fe(III), Mo(VI) and Pd(II) do interfere with the determination of nickel(II) when present in more than 2000 μg. The interference of Cu(II) can be eliminated by using 1.0 mL 0.2% oxalate as a masking agent. Fe(II) & Fe(III) are masked with 1.0 mL of 3.0% of sodium fluoride. The interference of Zn(II), Co(II) can be eliminated by using 1.0 mL of 0.5% of citrate solution. Anions like fluroride, thiocyanate, thiosulfate and thiourea do not interfere when present up to 3000 μg, with the determination of Ni(II) in the present developed method. Increasing masking agents proportionately can mask higher amounts of interfering ions.

Applications of the developed method

The developed extractive spectrophotometric method for nickel(II) has been successfully applied for its determination in medicinal leaves, soil and standard alloy samples.

Determination of Nickel (II) in soil samples

Soil samples were collected from in around Tirupati, AP, India. Each aliquot was analyzed for nickel (II) by the general procedure which was given in the experimental section, using 2, 6 – DAPTSC and the results obtained were checked by atomic absorption spectrometry (Table 2)

Determination of Nickel (II) in medicinal leaves

Medicinal leaves were collected from in and around Tirupati**,** AP, India. An appropriate amount of aliquot of each solution was analyzed for Nickel (II) by the recommended procedure given in the experimental section, using 2, 6 – DAPTSC and the results obtained were checked by atomic absorption spectrometry (Table 3)

Table - 1

Effect of Foreign Ions on the extraction of Ni(II)- 2,6-DAPBPTSC complex

Foreign Ion	Tolerance Limit, µg/ml	Foreign Ion	Tolerance Limit, µg/ml
As(III)	5500	V(V)	3000
As(V)	5500	Zn(II)	2000
Mn(II)	5500	Fe(III)	2000
Mg(II)	5500	Cu(II)	2000
Ca(II)	5500	Co(II)	2000
Sb(III)	5500	Thiocyanate	3000
Sr(II)	5500	Thiosulfate	3000
Ba(II)	5500	Thiourea	3000
Zr(IV)	5500	Fluoride	3000
Tl(III)	5500	Citrate	1500
Hg(II)	3000	Borate	1500
Bi(III)	3000		
Be(II)	3000		
Th(VI)	3000		
U(VI)	3000		
Al(III)	3000		

Table 2

Determination of Nickel (II) in some Soil Samples

Name of the area in Tirupati	Ni(II)[a] found µg, g^{-1}		Standard Deviation	Relative Standard Deviation%
	AAS Method	Proposed method		
Tiruchanur	24.5	23.9	0.230	0.962
Thondavada	20.10	19.97	0.170	0.855
Chandragiri	20.89	19.92	0.105	0.527
Renigunta	22.90	21.8	0.541	0.484
S.V. Nagar	22.50	21.95	0.003	0.015

[a] = average of four determinations

Table 3

Determination of Nickel (II) in medicinal leaves samples

Medicinal Leaves	Ni(II)[a] found µg g^{-1}		Standard Deviation	Relative standard Deviation%
	AAS Method	Proposed method		
Yerrajuvvi(Ficus microcarpa. L)	0.250	0.249	0.0010	0.401
Ramapala(Kalanchoe lanceolata)	0.348	0.349	0.0014	0.404
Mullagorinta(Barleria Prionitis. L)	0.401	0.402	0.0093	2.318
Gorinta(Lawsonia inermis. L)	0.211	0.212	0.0081	3.876
Jilledu(Calotropis gigantean. L)	0.278	0.279	0.0012	0.462
Ummetta(Datura stramonium. L)	0.621	0.620	0.0081	1.514
Kalabanda(Aloe vera. L)	0.539	0.540	0.0012	0.462

[a] = average of four determinations

Comparison of the present method with other reported extractive spectrophotometric methods for the determination of Nickel (II) (Table 4.)

Reagent	pH	λmax/ nm	$\epsilon_{max,} \times 10^4$/Lmol^{-1}cm^{-1}	M:L	Beer's law range law μg. mL^{-1}	References
1,3-Cyclohexanedione dithiosemicarbazone-monohydrochloride	8.5-10.0	430	2.5	1:3	0.23-1.88	3
5,5'Dimethyl-1,2,3-cyclo hexanetrione-1,2dioxime-3-thiosemicarbazone	9.0	383	-	-	Up to 9.2	4
Di-2-pyridyl ketone thiosemicarbazone	6.0-6.9	395	1.96	1:2	0.5-2.5	5
2-(3-Sulphobenzoyl) pyridinethiosemicarba-zone	6.0-10.0	400	2.0	1:2	-	6
Pyridoxal-4-phenyl-3-thio-semicarbazone	4.0-6.0	430	1.92	1:1	0.5-5.0	7
2,6-diacetylpyridine bis-4-phenyl-3-thiosemi-carbazone	4.5	400	1.44	1:1	0.587 to 5.87	Present method

Conclusions

It is noted from literature that many thio- and phenylthiosemicarbazones are used in the extractive spectrophotometric determination of nickel(II). In the present investigation, the authors introduced a new reagent, 2,6-DAPBPTSC to the field of extractive spectrophotometric determination of nickel(II). The reagent was found to be sensitive when compared to earlier reported reagents. The selectivity of the reagent was further improved by the use of proper masking agents to suppress the interference of diverse metal ions like Pd(II), Mo(IV), Co(II), Cd(II) and Cu(II). The results from the present developed method clearly demonstrate the usefulness 2,6-DAPBPTSC as an extracting agent for the determination of nickel(II) in soil and medicinal leaves samples.

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Mycosporine-like Amino Acids: Natural Bioactive Compounds Having Pharmaceutical Potentials

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ABSTRACT

Ultraviolet radiation (UVR) has pronounced detrimental effects on all the sun-exposed organisms, including humans, since it is responsible for various types of cellular photodamage. To counteract their adverse effects, cyanobacteria has developed the ability to synthesize certain natural bioactive photoprotective compounds such as mycosporine-like amino acids (MAAs) having pharmaceutical and biotechnological potentials. The topical application of MAAs formulation containing porphyra-334 (P-334) + mycosporine-glycine (MG) on the skin of hairless mice reduced the clinical signs of erythema and edema following UV-B irradiation. The formulation was also found to be effective in lowering the generation of reactive oxygen species and cytotoxic thymine dimer formation. Thus, these bioactive compounds may be better suited as natural sunscreens that may provide protection against UV-induced photoaging and skin cancer.

Keywords: Antioxidant, cyanobacteria, mycosporine-like amino acids (MAAs), ultraviolet radiation (UVR).

Introduction

Solar influx of highly energetic ultraviolet-B (UV-B, 280-315nm) radiation has increased on the Earth's surface due to continuing destruction of the ozone layer by anthropogenically released chemicals such as chlorofluorocarbons (CFCs), chlorocarbons (CCBs) and organobromides (OBMs) [1]. Although very small proportion of solar ultraviolet radiation (UVR) contribute to the total irradiance on the Earth's surface (UV-C; 0 %, UV-B; <1 % and UV-A; <7 %), this part of the solar spectrum is energetically highly active. These shorter wavelength radiations may have deleterious effects on all sun-exposed organisms, since it can be absorbed by a range of biomolecules leading to cellular photodamage.

Cyanobacteria are phylogenetically a primitive group of Gram-negative prokaryotes being the only bacteria to possess higher plant-type oxygenic photosynthesis [2]. They also have the capacity to metabolize CO_2, O_2, N_2 and H_2. Fossil evidence dates their appearance to the Precambrian era (around 3.5 x $l0^9$ years) [3]**.** Cyanobacteria are the largest and most widely distributed group of photosynthetic prokaryotes on Earth and as a group are thought to have survived from a wide spectrum of environmental stresses such as heat and cold shock, drought, salinity, nitrogen starvation, photooxidation, anaerobiosis, osmotic and ultraviolet radiation**.** Cyanobacteria are cosmopolitan in distribution and the colonization of oceans, lakes, rivers, hot springs and soils, and their presence as symbiotic organisms in fungi and plants demand high variability in adapting to diverse environmental factors [2]. Cyanobacterial populations hold an important place in both aquatic as well as terrestrial ecosystems, as the major biomass producer. Their inherent capacity to fix atmospheric N_2 with the help of the enzyme nitrogenise makes them potent natural biofertilizers [4]**.** They occupy a central position in the nutrient cycling and are valuable sources of various natural products of medicinal, pharmaceutical and industrial importance [5, 6].

Harvesting of solar energy for photosynthesis and nitrogen fixation simultaneously exposes these cyanobacteria to lethal doses of UV-B (280-315 nm) and UV-A (315-400 nm) radiations in their natural habitats. It may cause several harmful effects such as loss in growth, survival, pigmentation, motility, orientation, N_2 metabolism, DNA and protein etc. [7-11]. Any adverse effects of radiations on cyanobacteria will ultimately affect the productivity of the ecosystems. However, certain

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cyanobacteria synthesize photoprotective compounds such as mycosporine-like amino acids (MAAs) and scytonemin to counteract the damaging effects of harmful radiations.

MAAs are small (<400 Da), colorless, water-soluble compounds having strong UV-absorption maxima (310 to 362 nm), high molar extinction coefficients (ε = 28100-50000 M^{-1} cm^{-1}) and photostability against several abiotic stressors that provides evidence for their photoprotective role [12-14]. These compounds are known to dissipate absorbed energy as heat into their surroundings, without generating reactive oxygen species (ROS) [15]. MAAs are supposed to be synthesized via the first part of the shikimate pathway, where 3-dehydroquinate ((DHQ)) serves as a precursor [16, 17]. The relative protection against UV-B damage provided by MAAs depends on the species and location of the pigments therein, significant but limited protection has been reported for various cyanobacteria with MAAs located in the cytoplasm. In these cases, only 10-26 % of the photons are absorbed by the pigment implying that MAAs prevent 3 out of 10 photons from hitting cytoplasmic targets [18].

Materials & Methods

Isolation & characterization of the organisms

The test organisms *Nostoc* sp. HKAR-2 and *Scytonema* sp. HKAR-3 [19] were isolated from hot springs of Rajgir, India, by using standard microbiological techniques as described by Sinha *et al*. [20]. *Nostoc* sp. HKAR-2 (Fig. 1A) is a member of the family Nostocaceae characterized by frothy thallus, gelatinous, circinate trichomes, sheath absent, cells ellipsoidal and heterocystous. *Scytonema* sp. HKAR-3 (Fig. 1B) is an autotrophically growing heterocystous, firmly sheathed and filamentous cyanobacterium that has false branching in its filaments, a feature characteristic of the family Scytonemataceae.

Cultures were grown under axenic conditions in autoclaved liquid medium [21] without a nitrogen source in an air-conditioned culture room at a temperature 20 ± 2 °C and illuminated by cool white fluorescent light at an intensity of 12 ± 2 W/m^2. Cultures were hand shaken five times daily. To avoid contamination, cultures were sub-cultured at regular intervals and all the experiments were conducted from an exponentially growing cells..

Irradiation Source and Treatment Procedure

Exponentially growing suspension cultures were transferred into sterile 75 mm Petri dishes covered with 395 nm (for PAR treatment), 320 nm (for PAR+UV-A treatment) and/or 295 nm (for PAR+UV-A+UV-B treatment) cut-off filter foils (Ultraphan, UV Opak Digefra, Munich, Germany) to obtain the desired radiation regimes. The cultures in the Petri dishes were transferred onto a water bath placed over a slowly rotating shaker to avoid heating from radiation and self-shading, respectively. The whole set-up was placed inside a UV-chamber and the suspension

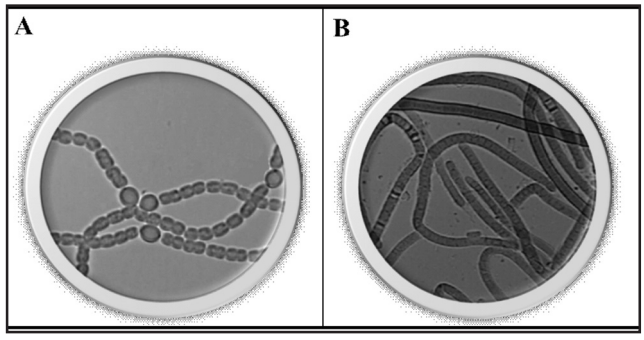


Fig.1 : Photographs showing the filaments of *Nostoc* sp. HKAR-2 (A) and *Scytonema* sp. HKAR-3 (B).

were exposed to artificial PAR, UV-A and UV-B produced from a PAR, UV-A and UV-B lamp, with the intensity of which extended from 280 - 400 nm of about 20 W/m^2, 5.0 W/m^2 and 2.0 ± 2 W/m^2 respectively.

Extraction of MAAs

Cyanobacterial cells were harvested by centrifugation and MAAs were extracted in 2.0 ml of 100 % (v/v) methanol (HPLC-grade) by overnight incubation at 4 °C. After extraction, the aliquots were centrifuged (10000 x g for 5 min) and supernatants (methanolic extracts) were evaporated to dryness at 45 °C and redissolved in 1 mL of double-distilled water and absorption spectrum was recorded. Thereafter, the samples were filtered through 0.2 m pore-sized microcentrifuge filters and subsequently subjected to HPLC analysis.

Absorption spectroscopy

Absorption spectra of all samples were measured in a double beam spectrophotometer (U-2910, UV/VIS, Hitachi, Tokyo, Japan). The raw data were transferred to a microcomputer and peaks were analysed with software provided by the manufacturer.

High-performance liquid chromatography

Analysis and purification of MAAs were done using a HPLC system (Waters 2998, Photodiode Array, pump L-7100, USA) equipped with a Licrospher RP 18 column and guard (5 μm packing; 250 mm × 4 mm inside diameter). The samples (50 μL) were injected into the HPLC column through a Waters 717 Plus Autosampler. The wavelength for detection was 330 nm, the mobile phase was 0.02 % acetic acid (v/v) in double-distilled water, at a flow rate of 1.0 ml min^{-1}. The MAAs were identified by comparing the absorption spectra and retention times with that of known MAAs.

Experimental animal

Hairless mice, aged 8–10 weeks housed under pathogen-free conditions, in controlled temperature (22 °C), humidity (60-70 %) and visible light (12 h light/12 h dark) were used. They were supplied with water and commercial mouse diet. The experiments were performed with the

approval of the Ethics Committee, Banaras Hindu University.

MAAs formulation and UV treatment

A water emulsion of MAAs formulation containing porphyra-334 and mycosporine-glycine was prepared to access their photoprotective role on mice-skin cells. Mice were anesthetized by intraperitoneal injection of the combination of ketamine and medetomidine prior to UV-treatment. The MAAs formulation at a concentration of 0.01µg ml^{-1} was topically applied on the dorsal skin surface of mice 20 min before irradiation. Untreated and exposed animals (NTI), as well as untreated and unexposed animals were used as controls. UV-B radiation at a dose of 0.75 Wm^{-2} was used for irradiation. Skin samples were collected after 0, 4, 8, 12, 24 and 48 h of exposure. The samples were subjected for photoprotective and antioxidative activity analysis in terms of free radical generation.

Histopathological, antioxidative and thymine dimer analyses

The formation of erythema and edema following UV-B exposure were assessed visually and evaluated by using the Draize scoring system [22] ranging from 0 (no erythema assigned to color of non-treated and non-irradiated skin) to 4 (highest redness corresponding to non-treated and irradiated skin). Edema values were 0: no edema, 1: slight edema (well-defined raising), 2: moderate edema (raised approximately 1 mm) and 3: severe edema (raised more than 1 mm). Production of H_2O_2 following UV-B exposure was accessed by absorption spectroscopy analysis at 260 nm. Genomic DNA was extracted from the skin samples after 24 h of UV-B exposure and thymine dimers were detected using the method as described earlier [23].

Results and Discussion

HPLC Chromatograms of partially purified aqueous solution from *Nostoc* sp. HKAR-2 revealed the presence of shinorine (RT - 2.2 min, λ_{max} - 334 nm), porphyra-334 (RT - 2.7 min, λ_{max} - 334 nm) (Fig. 2) and from *Scytonema* sp. HKAR-3 revealed the presence of mycosporine-glycine (RT - 4.0 min, λ_{max} - 310 nm) and unknown compound (RT - 8.5 min, λ_{max} - 334 nm) (Fig. 3).

Exposure to a UV dose of 0.75 Wm^{-2} induced significant erythema and edema in the non-treated and exposed animals (NTI) just 12 h after irradiation. Animals treated topically with P-334 + MG formulations showed a lower redness level. The initial erythemic response in P-334 + MG treatments decreased after 12 h and more than 50 % inhibition was observed after 48 h of treatment (Fig. 4). Similar results were observed in case of edema also. Signs of edema appeared in the exposed skin surface of experimental animal after 12 h of exposure and remained until the end of experiments. However, after the application of the formulation slight edema was observed (Fig. 5). Production of H_2O_2 following UV-B exposure was evaluated in the skin of mice by measuring its OD at 260 nm. A significant increase in the production of H_2O_2 was observed upto 4 h, but after that a slight decrease in the H_2O_2 production was observed. Topical application of the formulation was found to significantly inhibit the H_2O_2 production by almost 50 % after 12 h (Fig. 6). MAAs formulation also reduced the frequency of thymine dimer formation following UV-B exposure (Fig. 7).

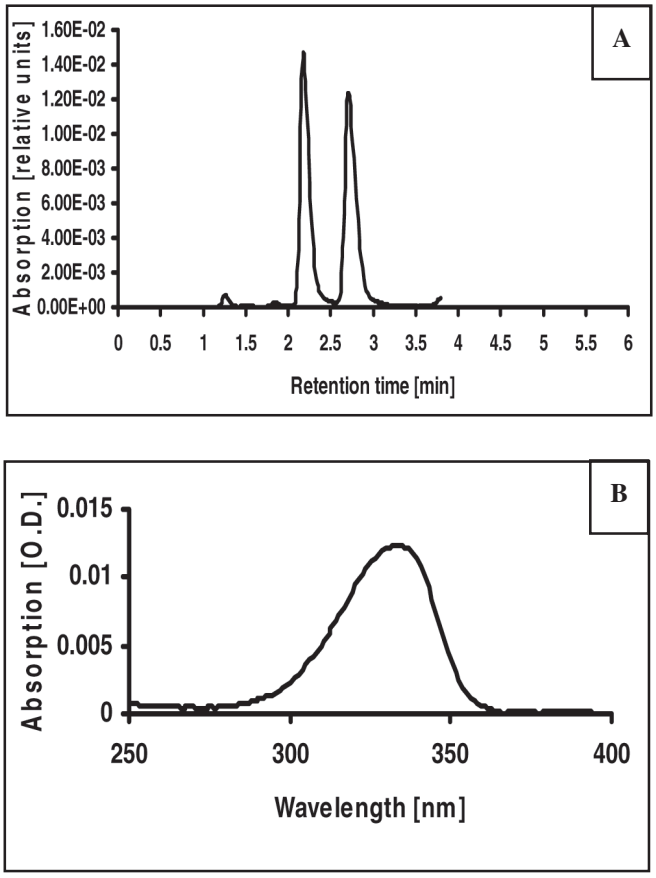
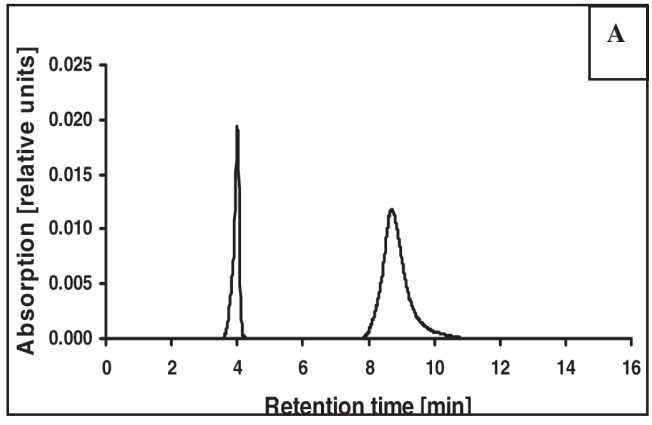


Fig. 2: Chromatogram and absorption spectrum obtained from the HPLC analysis of partially purified aqueous solution from *Nostoc* sp. HKAR-2. A. HPLC chromatogram of MAAs; B. Absorption spectrum of porphyra-334 (RT - 2.7 min, λ_{max} - 334 nm)



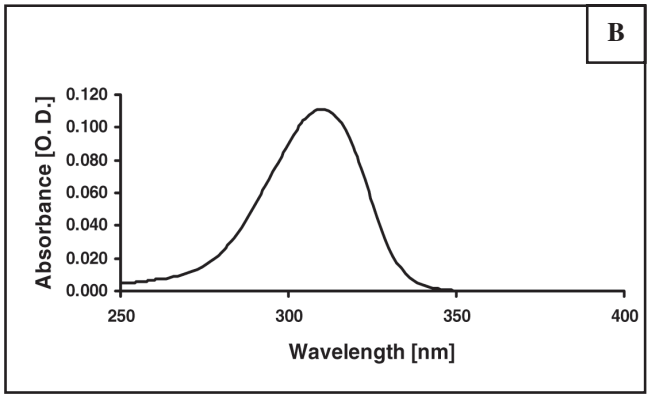


Fig. 3: Chromatogram and absorption spectrum obtained from the HPLC analysis of partially purified aqueous solution from *Scytonema* sp. HKAR-3. A. HPLC chromatogram of MAAs; B. Absorption spectrum of mycosporine-glycine (RT - 4.0 min, λ_{max} - 310 nm)

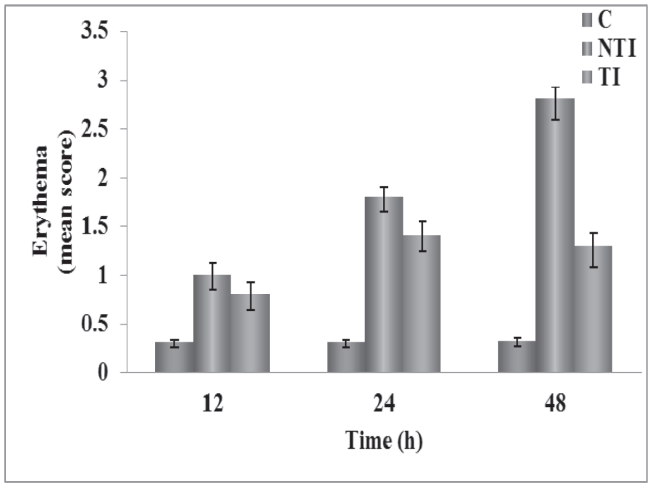


Fig. 4: Formation of erythema in mice skin cells after exposure to 0.75 Wm^{-2} UV-B radiation. Erythema formation was assessed visually and evaluated using the Draize scoring system, which ranges from 0 (no erythema assigned as control: non-treated and non-irradiated) to 4 (non-treated and irradiated skin).C: Control; NTI: non-treated and irradiated; TI: treated and irradiated.

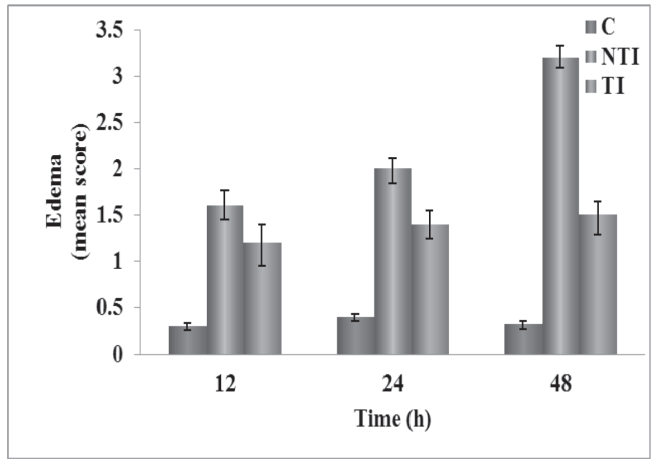


Fig. 5: Formation of edema in mice skin cells after exposure to 0.75 Wm^{-2} UV-B radiation. Edema formation was assessed using the Draize scoring system. C: Control; NTI: non-treated and irradiated; TI: Treated and irradiated.

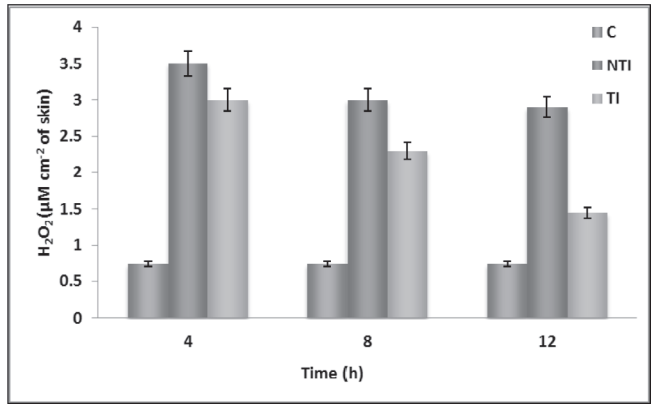


Fig. 6: MAAs mediated inhibition of UV-B induced H_2O_2 production in the skin of hairless albino mice. C: Control; NTI: non-treated and irradiated; TI: treated with the P-334 + MG formulation and then irradiated.

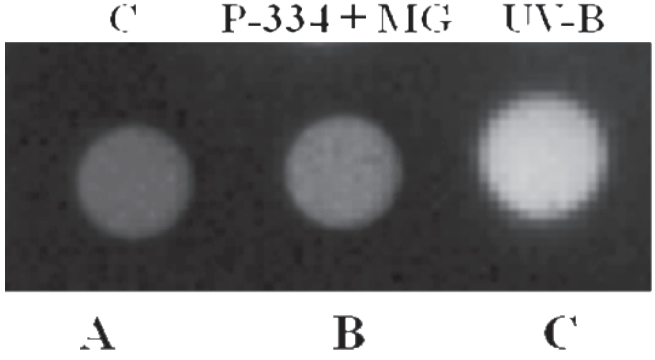


Fig. 7: Immunodot-blot assay of MAAs formulation treated and non-treated skin cells of mice. A – Control, B – after MAAs (P-334 + MG) treatment and C – untreated.

The present investigation showed the photoprotective role of natural bioactive MAAs combination, P-334 + MG in the skin of hairless mice exposed to UV-B radiation. The MAAs Porphyra-334 and mycosporine-glycine isolated from the cyanobacterial species *Nostoc* sp. HKAR-2 and *Scytonema* sp. HKAR-3 had an absorption maximum at 334 and 310 nm, respectively. The high molar extinction coefficients and photostability favours these MAAs as a potent natural sunscreen for photoprotection [24]. The *in vitro* test performed using a combination of MAAs, P-334+MG formulated in water showed to confer a broad spectrum of protection against UV-B radiation. Reactive oxygen species, such as H_2O_2, superoxide anion and singlet oxygen are thought to be involved in UV damage [25, 26]. Fortunately, the skin possesses a wide range of interlinked antioxidant defence mechanisms, e.g., catalase and SOD enzymes, to protect itself from damage by UV-induced ROS. In the present investigation, we have demonstrated that MAAs formulations are capable of reducing the generation of H_2O_2. MAAs exhibit a high antioxidant activity scavenging superoxide anions and inhibiting lipid peroxidation [27, 28] resulting from UV-induced production of ROS [29]. Recently, the antioxidant activity of P-334 and shinorine from *Porphyra* and other red algae, maintaining the antioxidant defense system of the skin as well as Hsp70 expression has been worked out by Coba et al. [30]. The antioxidant activities of the MAAs glycine and usujilene have been reported to inhibit lipid peroxidation in aqueous extracts of marine organisms and to scavenge $_1O^2$ generated from certain endogenous photosensitizers [29, 31]. Thus, it can be concluded that the mycosporine-like amino acids porphyra-334 and mycosporine-glycine can be considered as effective natural sunscreen substances, preventing the deleterious effects induced by ultraviolet radiation. The results suggest that in future these natural bioactive compounds may find application in cosmetic industries as a potent photoprotectant. Future research must be focused on the analysis, biosynthesis and mode of action of these natural UV-absorbing compounds.

Acknowledgement

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Solubility enhancement of Gliclazide with Poloxamer188 by lyophilization technique

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ABSTRACT

Gliclazide is an oral hypoglycaemic agent useful in the treatment of non-insulin dependent diabetes mellitus (NIDDM). It is practically insoluble in water and its absorption is dissolution rate limited. The present study was performed to improve the dissolution of Gliclazide, a poorly water soluble drug using solid dispersion technique. Thus, to enhance the dissolution, the hydrophilic carrier Poloxamer 188 in various proportions was prepared using lyophilization technique. Physical mixtures were also prepared. After comparing the solubility and dissolution profiles, it was observed that the solid dispersion of Gliclazide prepared by lyophilization technique with (1:5) showed the fastest dissolution. From the FTIR study it was conformed that there were no drug and polymer interactions. DSC studies revealed solid state interactions in solid dispersion as endothermic peak of Gliclazide was absent in the solid dispersions prepared by lyophilization technique indicating that the drug was in amorphous form. XRD study revealed presence of less number peaks indicating that that the drug was in amorphous form due to reduction in crystallinity.

Key words: solid dispersion (SDS), lyophilization technique, Poloxamer 188, dissolution.

Introduction

Gliclazide, 1-(1-azabicyclo [3,3,0]oct-3-yl)-3-p-tolylsulfonyl) urea is a second generation of hypoglycaemic sulfonylurea which is useful in the treatment of non-insulin dependent diabetes mellitus (NIDDM)[1]. Prior reports reveal that the drug shows good tolerability, low incidence of hypoglycaemia, and a low rate secondary failure[2]. Gliclazide is a white crystalline powder, relatively insoluble in water. Gliclazide exhibits slow GI absorption rate and inter individual variations of its bioavailability[3]. The slow absorption rate of drug usually originates from either poor dissolution of drug from the formulation or poor permeability of drug across GI membrane

The primary objective of the present study is to investigate the possibility of improving the release properties of gliclazide via lyophilization technique with poloxamer 188. The possible interactions between gliclazide and poloxamer 188 in both solid state and liquid states were investigated. Interaction in the solid state was investigated by Fourier-transform infrared (FT-IR) spectroscopy and differential scanning calorimetry (DSC). Interaction in solution was studied by phase solubility analysis and dissolution experiments.

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Materials and Methods

Gliclazide was obtained as a gift sample from Glenmark Generic Ltd., Mumbai (India) and Poloxamer 188 was obtained as a gift sample from B.A.S.F, Mumbai. All other reagents and solvents used were of analytical grade.

Preparation of SDs and physical mixture

Lyophilization technique:

The SDs of gliclazide in poloxamer 188 containing three different weight ratios (1:1, 1:2, 1:5) (gliclazide: Poloxamer 188) and denoted as SD1/1, SD1/2 and SD 1/5 respectively, were prepared by lyophilization. The model drug and the hydrophilic polymer Poloxamer 188 solubilized in approximately 20 ml of isopropanol in small USP type glass vial (30ml). Then the vials were kept into the port of Yorco Freeze Dryer, YSL-250 in close condition of valve. After the stabilization lyophilizer the ports were opened slowly and kept in that condition for several hours until powder mass was obtained .Then the valves of lyophilizer were closed and the resultant powder mass pass through sieve no#60 and kept in the desiccator.

Physical Mixtures

The PMs having the same weight ratio as SDs were prepared by thoroughly mixing the required amount of gliclazide and poloxamer 188 for 10 min in a mortar. The resulting mixtures were sieved through a 100-mesh sieve and denoted as PM 1/1, PM 1/2 and PM 1/5 respectively.

Characterizations

Solubility determinations were performed in triplicate according to the method of Higuchi and Connors.Dissolution studies of gliclazide in powder form, SDs, and PMs were performed by using the U.S.Pharmacopoeia (USP) model digital tablet dissolution test apparatus-II at the paddle rotation speed of 50 rpm in 900 mL 0.1N HCl containing 0.25% (w/v) of SLS as a dissolution medium at 37±0.5°C at 276 nm.

Results and Discussion

Solubility Studies

Solubility experiments showed that the concentration of gliclazide in 0.1N HCl is notably affected by the presence of poloxamer 188. The phase-solubility diagram investigated in 0.1N HCl (pH 1.2) was linear up to concentrations of 1.5 (w/v) and correspond to A_N-type profiles (Fig 1).The apparent stability constant ($K_{1:1}$) was estimated from the slope of the straight line of the phase-solubility diagram. The stability constant was found to be 0.0231 ml^{-1}mg. These results are in accordance with the well established formation of soluble complexes between water soluble polymeric carriers and poorly water soluble drugs.

Dissolution Studies

The results of the dissolution studies for individual samples (gliclazide alone, PMs and SDs) over the period of 1 hour are shown in Fig 2 and reported values are the mean three determinations (CV<10%).Q_{10}, Q_{20} and Q_{30} values (percent drug dissolved within 30 minutes) are shown in fig 2. From the fig 2 it is evident that onset of dissolution of pure gliclazide is very low about 40.44% of drug being dissolved within 30 minutes. SDs of gliclazide with poloxamer 188 considerably enhanced dissolution rates within 30 minute compared to pure gliclazide and PMs.

The value of $\%DE_{10min}$ for pure gliclazide (9.16%) was enhanced in PMs (36.90%) as well as in SDs (40.02%). The value of $\%\ DE_{30min}$ for the pure drug was increased to 64.9 % in PMs and up to 67. 39 % in SDs.

The MDT of gliclazide is 12.5 min, then it decreased to a greater extent 5.24 min after preparing its SDs with poloxamer 188 at 1:2 (gliclazide: poloxamer 188) ratio. The MDT of gliclazide increases with increasing concentration of poloxamer 188 in its PMs as well as SD.

Table.1

Effect of poloxamer 188 concentration and Gibbs free energy on solubility gliclazide

Concentration of poloxamer 188 (% w/v)	Concentration of gliclazide(mg/ml) at 37 °C	$\Delta\Delta G_{tr}^{o}$(J/Mol)
0	0.81	0
2.0	0.92	-332.98
2.5	0.99	-521.87
3.0	1.4	-1415.15
3.5	1.8	-2063.0
4.0	2.1	-2460.38

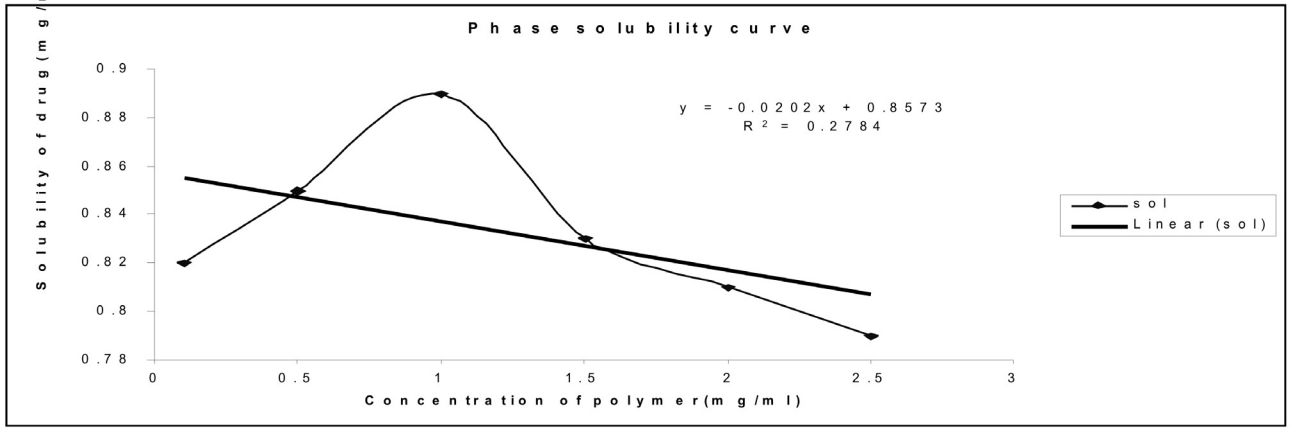

Fig.1 : Phase solubility curve

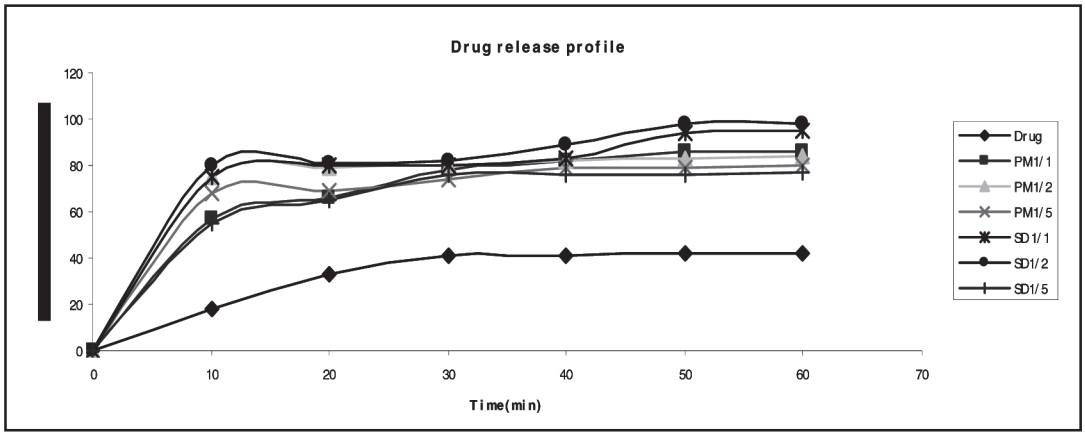

Fig.2 : In-*vitro* dissolution profile of gliclazide, physical mixture of gliclazide and solid dispersion of gliclazide with poloxamer 188 in 0.1NHCl (pH 1.2)

Table - 2

***In-vitro* dissolution profile of gliclazide, physical mixture of gliclazide and solid dispersion of gliclazide with poloxamer 188 in 0.1NHCl (pH 1.2)**

Formulations	Dissolution parameters					
	Q_{10min}	Q_{20min}	Q_{30min}	$\%DE_{10min}$	$\%DE_{30min}$	MDT(min)
Drug	18.46	32.67	40.82	9.16	23.67	12.5
PM 1:1	56.6	65.6	78.0	28.32	53.75	9.32
PM 1:2	61.6	67.2	73.2	33.78	57.96	6.57
PM 1:5	62.3	68.2	74.12	36.90	64.90	5.75
SD 1:1	67.2	69.7	75.2	33.58	58.15	6.80
SD 1:2	75.15	79.77	80.29	37.58	65.03	5.7
SD 1:5	80.0	81.3	81.6	40.02	67.39	5.24

Table-3

Statistical parameters of various formulations of gliclazide with pol-188 after fitting drug release data to various release kinetics models

Formu-lations	Zero-order Model		First-order Model		H-M Model		P-K Model		H-C Model	
	R	K_1	R	K_2	R	k_3	R	$K_{4,n}$	R	K_5
Drug	**0.9813**	1.4736	**0.9939**	-0.0184	**0.9893**	7.1358	**0.9952**	3.473, 0.724	**0.9905**	-0.0057
PM 1:1	**0.6131**	1.8651	**0.9001**	-0.0397	**0.9460**	12.8338	**0.9830**	31.76, 0.254	**0.8272**	-0.0100
PM 1:2	**0.765**	1.892	**0.3554**	-0.0398	**0.7990**	13.283	**0.9909**	65.70, 0.060	**0.1212**	-0.0101
PM 1:5	**0.0869**	1.7808	**0.5998**	-0.0344	**0.8514**	12.4232	**0.9613**	51.92, 0.107	**0.4796**	-0.0090
SD 1:1	**0.3371**	2.0303	**0.8856**	-0.0531	**0.8760**	14.096	**0.9042**	54.46, 0.128	**0.7638**	-0.0121
SD 1:2	**0.3370**	2.1160	**0.9351**	-0.0696	**0.8739**	14.6880	**0.8682**	57.53, 0.124	**0.8421**	-0.0141
SD 1:5	**0.3581**	2.523	**0.9452**	-0.0741	**0.8842**	15.123	**0.8785**	58.25, 0.131	**0.8514**	-0.0156

*H-M indicates, Highuchi Matrix; †P-K, Peppas-Korsmeyer; ‡H-C, Hixon-Crowell; §R indicates correlation coefficient; § K_1- K_5, Constants of release kinetics; PM: Physical mixture with poloxamer; SD: Solid dispersion of gliclazide with poloxamer.

FTIR-Spectroscopy

Fourier-transform infrared (FT-IR) spectroscopy was used to characterize possible interactions between the drug and carrier in the solid state. The IR spectra of SDs and PMs were compared with the standard spectrum of gliclazide.IR spectrum of gliclazide is characterized by the absorption of carbonyl (C=O) sulphonyl urea group at 1706 cm^{-1}. In spectra of SDs and PMs, this band was shifted towards higher frequencies at 1725 cm^{-1} and 1711 cm^{-1} respectively. Also the NH group which is located at 3265 cm^{-1} from the IR spectrum of gliclazide shifted to 3365 cm^{-1} in SDs. The sulphonyl group bands are located at 1349 cm^{-1} and 1162 cm^{-1} in pure gliclazide. In SDs, the asymmetrically vibration peak of S=0 band was shifted from 1349 cm^{-1} to 1341 cm^{-1} with decreased frequencies. In SDs, the symmetrically stretching vibration band of S=0 was shifted from 1162 cm^{-1} to 1157 cm^{-1} with decreased frequencies. The shift in the peaks associated with sulfonylurea group of the gliclazide indicate an increase in bond strength possibly due to stabilizing effect of the hydrogen atoms of poloxamer 188 interacting with the oxygen atoms of the sulphonyl group. Mentioned evidences thus lead to the conclusion that changes seen are as a result of physical interaction between the gliclazide and poloxamer 188. Although it could be expected to have hydrogen bonding between the hydrogen atom of the NH group of gliclazide and one of the ion pairs of oxygen atom in the poloxamer 188, that could not be demonstrated.

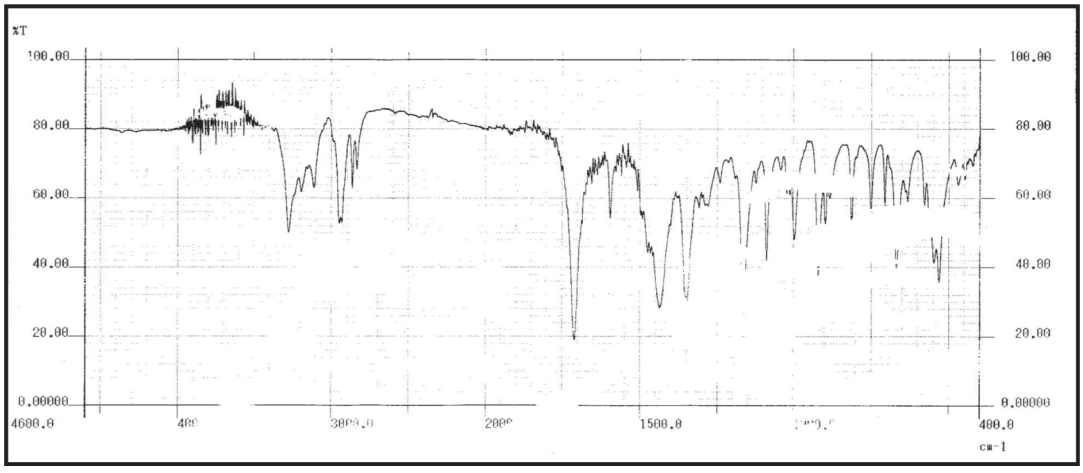

Fig.3: Infrared spectra of Gliclazide

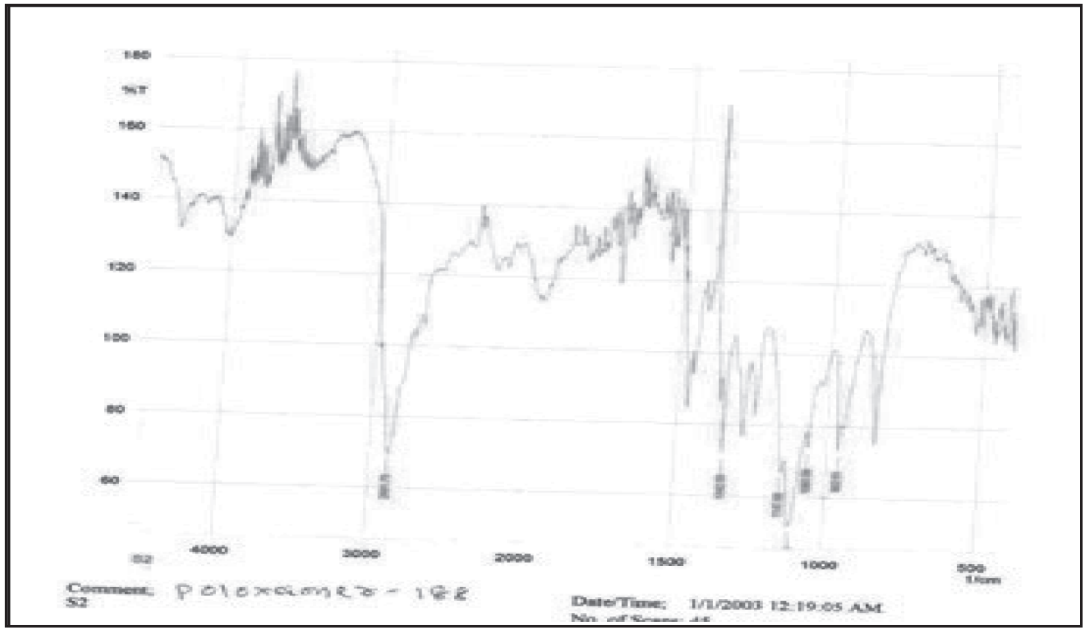

Fig.4 : Infrared spectra of Poloxamer-188

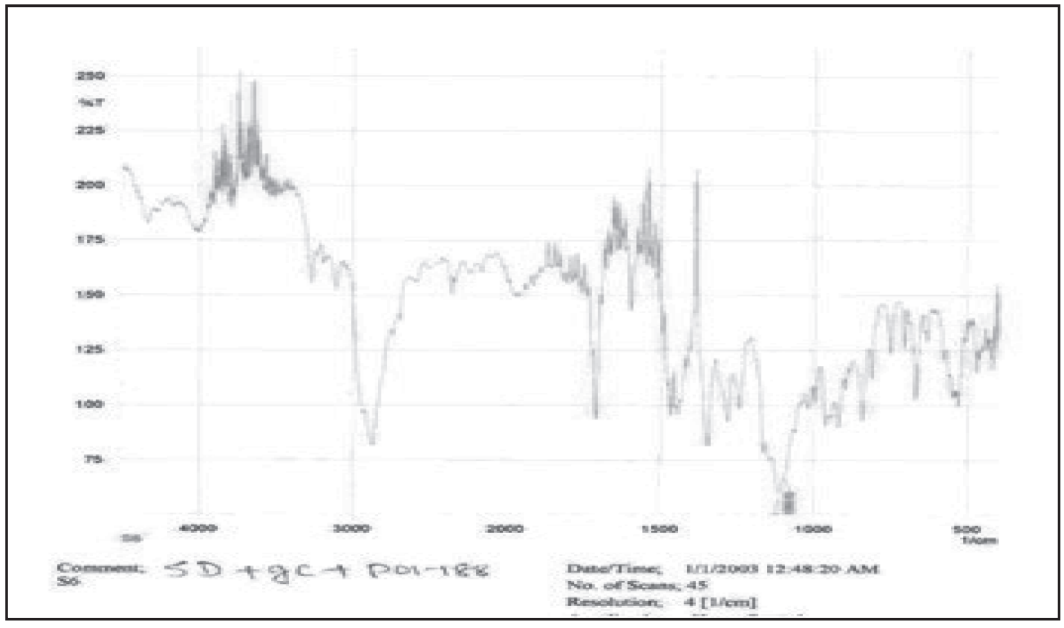

Fig.5 : Infrared spectra of Physical mixture of Gliclazide -poloxamer188

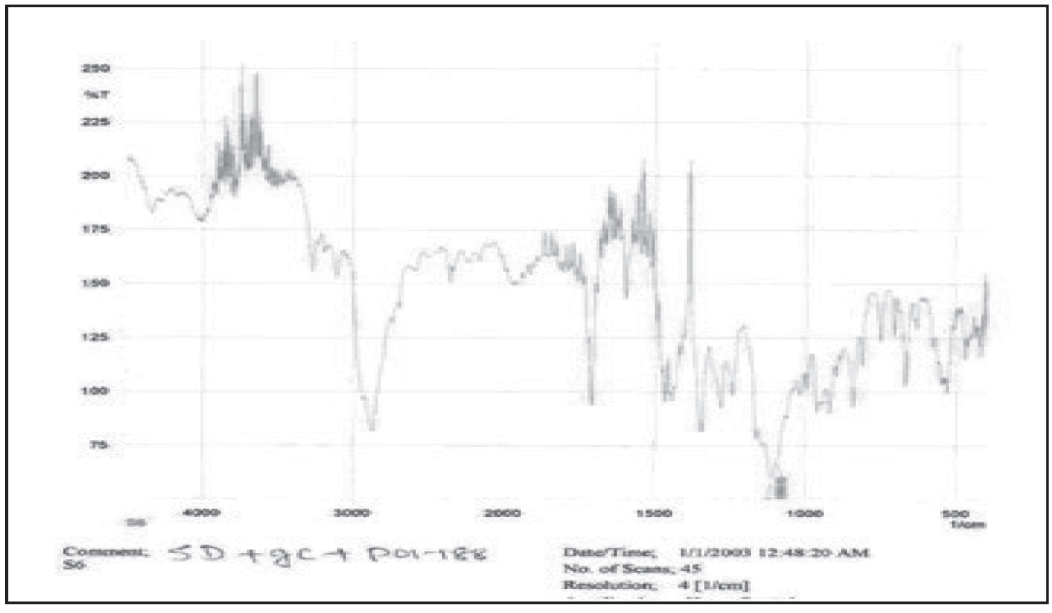

Fig.6: Infrared spectra of Solid dispersion of Gliclazide -poloxamer188

Differential Scanning Calorimetry

The DSC curve of pure gliclazide exhibited a single endothermic response corresponding to the melting of drug. Onset of melting was observed at 170.8ÚC, the corresponding heat of fusion (ΔH_F) was 171.8J/g where as pure poloxamer 188 showed a melting endotherm at 58.2ÚC corresponding heat of fusion (ΔH_F) was 259.5J/g. Thermogram of SDs showed the absence of a gliclazide peak, suggesting that gliclazide is completely soluble in the liquid phase of polymer. However, the melting peak of poloxamer 188 in SDs was observed at slightly lower temperature (57.2ÚC) than that of pure poloxamer 188.The PMs Formulation of gliclazide and poloxamer 188 also showed no endothermic peak of gliclazide, even though the peaks derived from gliclazide were observed in XRD. It is speculated that gliclazide dissolved in melted poloxamer 188 during the DSC measurement, only one endothermic peak at 57ÚC corresponding to melting of poloxamer 188 was observed.

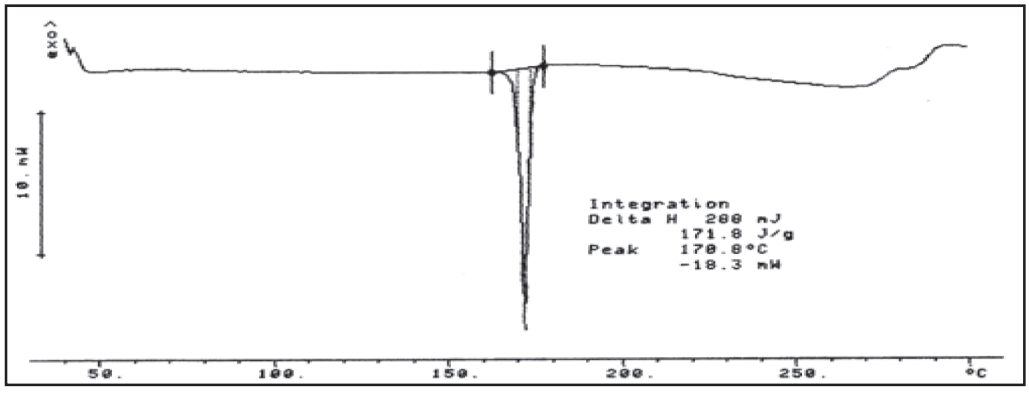

Fig.7 : Gliclazide DSC

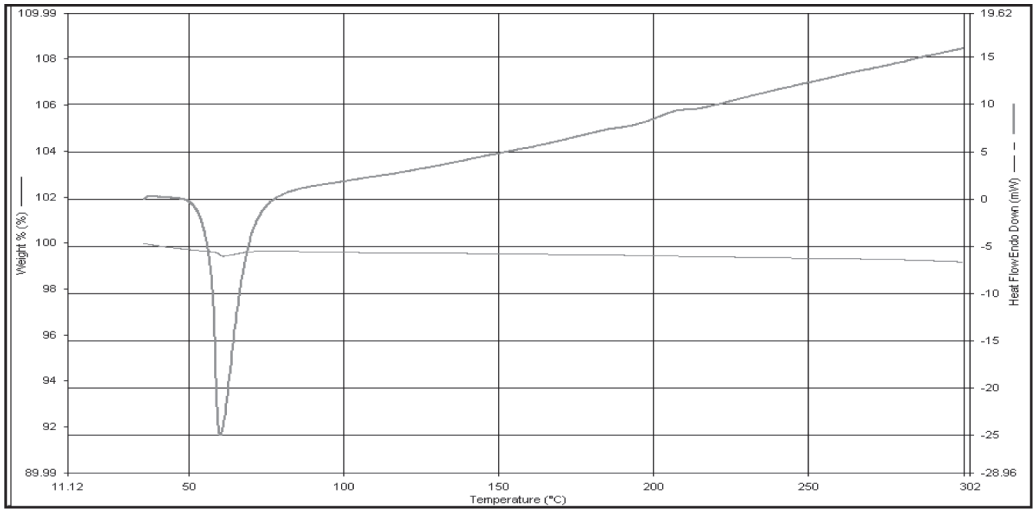

Fig.8 : Poloxamer 188 DSC

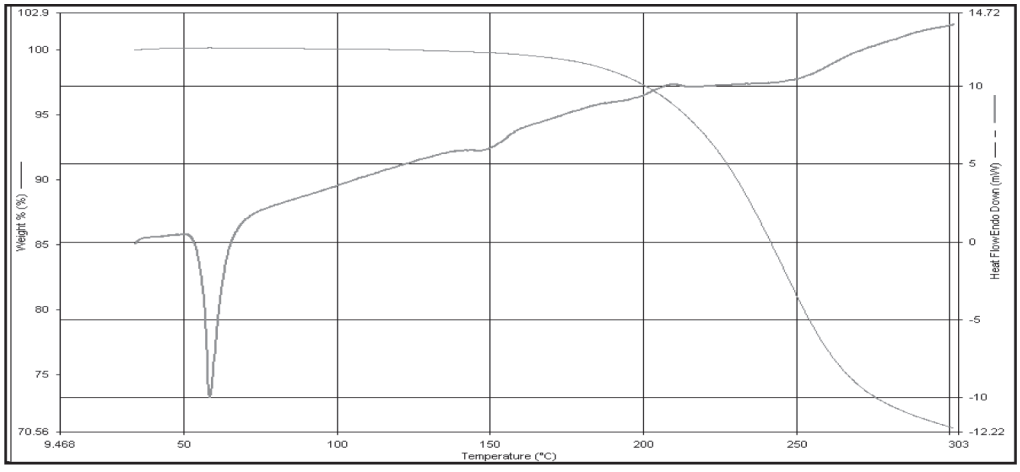

Fig.9 : DSC thermogram of PM (Gliclazide +Pol 188)

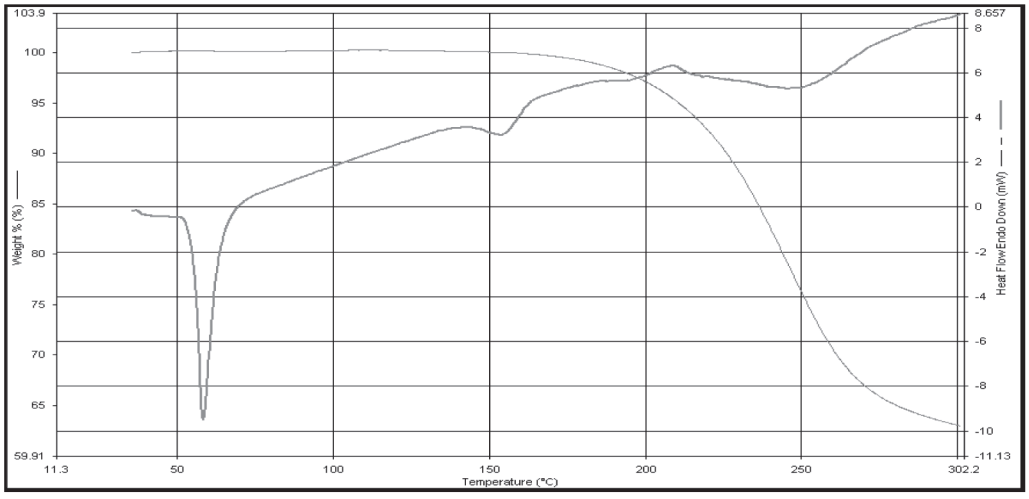

Fig.10: DSC thermogram of Solid dispersion (Gliclazide +Pol 188)

Conclusion

The solubility and dissolution rate of gliclazide can be enhanced by the use of SDs of gliclazide with poloxamer 188 by lyophilization technique. The solubilization effect of poloxamer 188, reduction of particle aggregation of the drug, absence of crystallinity, increased wettability and dispersibility, and alteration of the surface properties of the drug particles might be responsible for the enhanced solubility and dissolution rate of gliclazide from its SD and PMs.

Acknowledgement

Author wish to thank the management of Anwarul Uloom College of Pharmacy, New Mallepally, Hyderabad for providing the facility to carry out research work.

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   Fig1 Effect of concentrations of poloxamer 188 on solubility of gliclazide
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Synthesis and characterization of metal complexes of 4-aminobenzene sulfonamide Schiff base

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ABSTRACT

A novel Schiff base formed by the condensation of 2-hydroxybenzaldehyde with 4-aminobenzene sulfonamide and its Fe(III), Ru(III), Co(II), Ni(II), Cu(II), Pd(II), Zn(II), Cd(II) and Hg(II) complexes have been prepared and structurally characterized by various physico-chemical data. The ligand acts as a mononegative bidentate one towards the metal ions coordinating through phenolic oxygen and azomethine nitrogen. The geometry and the bonding characteristics associated with the complexes have been deduced from relevant spectral data. All the complexes are coloured and stable towards air and moisture. The complexes exhibit coordination numbers of 4 and 6.

Introduction

Preparation of new ligands is perhaps the most important step in the development of metal complexes that exhibit unique desired properties and novel reactivity. The chemistry of metal complexes with tailor-made Schiff base ligands and their applications have aroused considerable interest, mainly because of their preparative feasibility and structural variability. Metal complexes of Schiff bases have played central role in the development of coordination chemistry [1], analytical chemistry [2] and pigments, dyes [3] and polymer [4,5] industries; in biochemical front, they serve as model compounds of several vitamins and enzymes [6,7] and in agriculture as fungicides, pesticides and bactericides [8,9]. Partaking interest in this regard, we report, herein, the synthesis and characterization of a schiff base: 4-((2-hydroxy benzylidene) amino) benzene sulfonamide (HBABS; Fig.1) and its Fe(III), Ru(III), Co(II), Ni(II), Cu(II), Pd(II), Zn(II), Cd(II) and Hg(II) complexes.

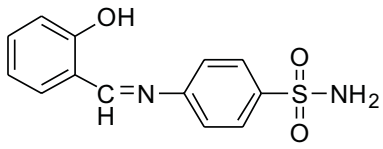

Fig.1 : HBABS

**Address for correspondence*

Expermental

All the chemicals used were of AR or BDH grade. The ligand HBABS was prepared by refluxing an equimolar mixture of 2-hydroxybenzaldehyde and 4-aminobenzenesulfonamide in methanol in presence of a few drops of concentrated H_2SO_4 for about 3 hrs. The solid that separated was filtered, washed with water and recrystallised from methanol. The colour, yield (%), m.p. and elemental analysis (%) of HBABS are respectively yellow, 56, 180^{o}c [found C,55.52; H,3.88; N,9.30]

The Fe(III), Ru(III), Co(II), Cu(II), Pd(II) and Hg(II) complexes of ligands were prepared taking metal chlorides and Ni(II), Zn(II) and Cd(II) complexes taking metal acetates. In the preparation of metal complexes, the metal and the ligand were combined in1:3 mole ratio in the case of Fe(III) and Ru(III) and in 1:2 mole ratio in the case of Co(II), Ni(II), Cu(II), Pd(II), Zn(II), Cd(II) and Hg(II) using required quantities of methanol so as to effect the solubility of the metal salts and the ligand. The contents were refluxed on a hot water bath for 2-3 hrs and the solid that separated was filtered, washed with water, hot methanol and ether and was vacuum dried over fused $CaCl_2$.

The elemental analyses(C,H,N) of the ligand and the complexes were carried out at C.S.M.C.R.I., Bhavanagar. Conductance measurements were made in DMF at 10^{-3} M concentration on a Digisun digital conductivity meter. Gouy balance calibrated with $Hg[Co(NCS)_4]$ was used to measure

the magnetic susceptibility of metal complexes at room temperature. The IR spectra of the ligand and its metal complexes in KBr were recorded in the range 4000-450 cm^{-1} using Perkin Elmer 100 FT-IR spectrophotometer. The electronic spectra of metal complexes were recorded on Perkin Elmer UV-VIS spectrophotometer. WIN-EPR (BRUKER) spectrophotometer operating in the frequency 9.5 GHz was employed in recording the ESR spectrum of Cu(II) complex in DMF at LNT.

Results and Discussion

The Fe(III), Ru(III), Co(II), Ni(II), Cu(II), Pd(II), Zn(II), Cd(II) and Hg(II) complexes of HBABS are stable at room temperature and are non-hygroscopic. Upon heating, the complexes decompose without melting. The complexes are insoluble in water, slightly soluble in hot methanol and fairly soluble in dimethylformamide and dimethylsulphoxide.

The elemental analyses (Table 1) show that the Fe(III) and Ru(III) complexes have 1:3 and the Co(II), Ni(II), Cu(II), Pd(II), Zn(II), Cd(II) and Hg(II) complexes 1:2 metal-ligand stoichiometry. All the complexes are non-electrolytic in nature [10].

IR Spectral Data

The ligand shows, in its IR spectrum, a medium intensity band at 3345cm^{-1} that has been assigned to νO-H. This band disappears in the spectra of its complexes indicating that deprotonation of the group has taken place. A small intensity band at 1239cm^{-1} in the ligand assignable to νC-O is seen to have undergone a positive shift by 20-40cm^{-1} in the complexes suggesting coordination through phenolic oxygen [11]. A band that shows up at 1615cm^{-1} in the ligand due to azomethine group has been lower shifted by 15-20 cm^{-1} in the complexes indicating that the nitrogen of this group is coordinated to the metals [12]. The presence of SO_2 group in the ligand is indicated by two bands around 1160 and 1370 cm^{-1} due to its symmetric and asymmetric stretching vibrations respectively and they remain unshifted in the complexes indicating that the SO_2 group is not involved in coordination [13]. These observations suggest that the ligand acts as a mononegative, bidentate one bonding through phenolic oxygen and nitrogen of C=N group, [14,15].

The coordination through phenolic oxygen and azomethine nitrogen is further substantiated by the appearance, in all the complexes, of non-ligand bands in the far infrared region around 520 and 460cm^{-1} assignable respectively to νM-O and νM-N vibrations[16].

Electronic Spectral Data

The electronic spectral data of the Ru(III), Co(II), Ni(II), Cu(II) and Pd(II) complexes along with the assignment are furnished in Table-2.

These transitions are characteristic of octahedral (low spin) for Ru (III), tetrahedral for Co(II) and square planar geometry for Ni(II), Cu(II) and Pd(II) complexes [17-19].

Table-1

Analytical and physical data of the metal complexes

Metal complex	Colour	Per cent			Molar Conductance $\Omega^{-1}cm^2mol^{-1}$	μ_{eff} B.M.
		C	H	N		
[Fe $(C_{13}H_{11}N_2O_3S)_3$]	Brown	52.24(53.12)	3.28(3.77)	9.20(9.53)	12	5.58
[Ru $(C_{13}H_{11}N_2O_3S)_3$]	Brown	54.62(55.53)	3.16(3.59)	8.86(9.07)	14	1.81
[Co $(C_{13}H_{11}N_2O_3S)_2$]	Brown	50.66(51.23)	3.28(3.64)	8.78(9.19)	12	4.50
[Ni $(C_{13}H_{11}N_2O_3S)_2$]	Pale green	50.72(51.25)	3.30(3.64)	8.92(9.20)	10	-
[Cu $(C_{13}H_{11}N_2O_3S)_2$]	Brown	50.04(50.85)	3.18(3.61)	8.78(9.12)	13	1.78
[Pd $(C_{13}H_{11}N_2O_3S)_2$]	Grey	46.86(47.53)	3.09(3.38)	8.02(8.53)	14	-
[Zn $(C_{13}H_{11}N_2O_3S)_2$]	Light Yellow	49.80(50.69)	3.22(3.60)	8.90(9.10)	12	-
[Cd $(C_{13}H_{11}N_2O_3S)_2$]	Yellow	46.68(47.10)	3.08(3.34)	8.02(8.45)	11	-
[Hg $(C_{13}H_{11}N_2O_3S)_2$]	Yellow	40.76(41.57)	2.81(2.95)	7.00(7.46)	10	-

Values in parentheses are the calculated ones.

Table-2
Electronic spectral data of the metal complexes.

Complex	Frequency (cm^{-1})	Assignment
Ru-HBABS	16650	$^2T_{2g} \rightarrow {}^4T_{1g}$
	18410	$^2T_{2g} \rightarrow {}^4T_{2g}$
	22600	$^2T_{2g} \rightarrow {}^2A_{2g}$
Co-HBABS	11900	$^4A_2(F) \rightarrow {}^4T_1(F)$
	20000	$^4A_2(F) \rightarrow {}^4T_1(P)$
Ni-HBABS	17700	$^1A_{1g}(D) \rightarrow {}^1B_{1g}(G)$
	24100	$^1A_{1g}(D) \rightarrow {}^1E_g(G)$
Cu-HBABS	15625	$^2B_{1g} \rightarrow {}^2B_{2g}$
	20830	$^2B_{1g} \rightarrow {}^2E_g$
Pd-HBABS	15150	$^1A_{1g} \rightarrow {}^1A_{2g}$
	19610	$^1A_{1g} \rightarrow {}^1B_{1g}$
	25640	$^1A_{1g} \rightarrow {}^1E_g$

The Fe(III) complex, owing to transitions in it being spin-forbidden and the Zn(II), Cd(II) and Hg(II) complexes due to filled configurations, do not show d-d bands. Based on the other data obtained, Fe(III) complex has been assigned octahedral (high spin) and the other three complexes, tetrahedral geometry.

ESR Spectral Data

The ESR spectrum of Cu(II) complex is of anisotropic nature in that it shows two peak envelopes, one of small and the other of large intensities. The $g_{\parallel}$ and $g_{\perp}$ corresponding to these peaks are 2.22 and 2.05 with a $g_{av} = (g_{\parallel} + 2g_{\perp}/3) = 2.11$.

The anisotropic nature of the spectrum provides an additional evidence for square planar geometry of the complex. In a square planar geometry, the unpaired electron lies in $d_{x^2-y^2}$ orbital giving $^2B_{1g}$ as the ground state with $g_{\parallel} > g_{\perp} > 2$ [20, 21]. Conforming with this, it may be inferred that the unpaired electron lies predominantly in the $d_{x^2-y^2}$ orbital of Cu(II).

Conclusions

Based on the foregoing discussion, it may be concluded that the ligand acts in a mononegative, bidentate manner coordinating to the metals through phenolic oxygen and azomethine nitrogen. Fe(III) and Ru(III) complexes are octahedral, Ni(II), Cu(II) and Pd(II) complexes, square planar and Co(II), Zn(II), Cd(II) and Hg(II) complexes, tetrahedral in geometry .

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Impact of Calcium and Magnesium Ions in Identification of Second Generation Offspring Gender in rabbits

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ABSTRACT

Impact of calcium and magnesium in diet to determine offspring gender in rabbits has been investigated. Sex chosen from the second generation of the previous experimented rabbits were divided into two groups in the ratio male to female 1:1. The first group was Ca and Mg, the second group determination has scientific basis for prevention of genetic diseases in addition to social backgrounds. 20 healthy rabbits was control unit without Ca and Mg. It was found that the delivered offspring male to female ratio were 1.8:1 and 1:1 for the first and second groups respectively. Also, it was found that rabbits fed with (Ca, Mg) food yields maximum numbers of female offspring 45, while rabbits fed with normal food yields lowest numbers of female offspring 35.

Keywords: *Calcium, Magnesium, Sex ratio, Rabbits, Offsprings.*

Introduction

Pregnancy is a state that allows a life form to develop with the support and protection from mother's body. The growth and development of the fetus in gestation is partially determined by the genome of the fetus, which produces its own growth factors as well as the majority of its hormones. However, this genetic influence is highly dependent upon interaction with environmental factors [1]. One environmental factor vital in the growth and development of the fetus is nutrition. A balanced, nutritious diet is an important aspect of a healthy pregnancy. Eating a healthy diet, balancing carbohydrates, fat, and proteins, and eating a variety of fruits and vegetables, usually ensures good nutrition. Those whose diets are affected by health issues, religious requirements, or ethical beliefs may choose to consult a health professional for specific advice. Adequate periconceptional folic acid (also called folate or Vitamin B_9) intake has been proven to limit fetal neural tube defects, preventing spina bifida, a very serious birth defect. The neural tube develops during the first 28 days of pregnancy, explaining the necessity to guarantee adequate periconceptional folate intake[2,3].Folates are abundant in spinach (fresh, frozen, or canned), and are found in green leafy vegetables e.g. salads, beets, broccoli, asparagus, citrus fruits and melons, chickpeas and eggs. In the United States and Canada, most wheat products (flour, noodles) are fortified with folic acid [4].

DHA omega-3 is a major structural fatty acid in the brain and retina, and is naturally found in breast milk. It is important for the woman to consume adequate amounts of DHA during pregnancy and while nursing to support her well-being and the health of her infant. Developing infants cannot produce DHA efficiently, and must receive this vital nutrient from the woman through the placenta during pregnancy and in breast milk after birth [5].

The fetus is solely dependent on the mother to supply its nutrients. It is also dependent on the placenta, an essential organ in pregnancy, to transfer these nutrients from the maternal system to its own. Thus the fetal nutrition is a reflection of that of the mother's. This interaction exists in a sensitive equilibrium; if disturbed, there are fetal developmental consequences[1].Preselection of the gender of offspring is a subject that has held man's attention since the beginning of recorded history. Although scientific studies on genes have been conducted recently, sex selection and gender preference have been considered since ancient time.

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Anaxagoras, a greek scientist was the first person who related the sex of fetus to testis [6].

There are many Methods of sex selection such as: The consumption of particular foods, the use of various vaginal douches and the timing of intercourse in relation to ovulation, Sperm sorting, Pre-implantation genetic diagnosis (PGD), Selective abortion, Infanticide, Periconceptual methods, postconceptual methods.

There are also methods which use different food combinations and especial diets to maximum the chance of having a baby with specific sex. The old believe is that eating salty, savory foods leads to delivering a male and sodium rich foods to a girl. Some believes that the ratios of the minerals sodium, potassium are important in determination of offspring gender. It was shown that pregnant female house mice maintained on a consistent low-food diet give birth to a lower proportion of males than do control females fed ad libitum [7].

As a part of our ongoing research, we studied the Role of Sodium and Potassium ions in identification of offspring gender in mammals [8,9,10,11,12,13,14,15and16].

In this study, we study the effects of adding bivalent ions (calcium and magnesium) to the drinking water of rabbits, offspring sexes was investigated.

Materials and Methods

20 Adult female second generation of rabbits weighting 1100-1300 g, 9 months old and still in their reproductive phase, were kept in metabolic cages individually and separately and within 16-21 days, on the specified diets (Ca, Mg and non Ca, Mg) feeding and metabolism control .The first group (Ca, Mg) was supplied with drinking water mixed with 1% sodium and potassium, the second group was chosen as a control group without Ca, Mg, pure drinking water was supplied. After 16-21 days, on the specified diets, the rabbits at the stage of the reproductive cycle were caged with male rabbit for mating and gestational 10 minutes. When rabbit is pregnant that it may start rejecting advances mate the male rabbit. The number of litters and the gender of pups were recorded. Pups were sexed by means of the ano-genital distance, which is longer in males[14]; this was confirmed in later examinations during pre weaning development. The data were entered and analyses by SPSS software using t.test and the p-value less than 0.05 were considered as significant.

Results and Discussion

It was found that, in the first group mothers (Ca, Mg); all of the 10 rabbits became pregnant which delivered 70 offspring. Their gender was 25 male 35.71% and 45 female 64.29%. In the second group, Non (Ca, Mg); all of the10 rabbits became pregnant and delivered 70 offspring, their gender was 35 male 50% and 35 female 50% (Table 2, Figure 2).

The sex ratio of female to male in the first group of mothers (Ca, Mg) was 1.8:1, While this ratio in the second group, Non (Ca, Mg) was1:1 respectively (Table 2).The percentage of the female offspring of mothers (Ca, Mg) 64.29 % was higher than the female offspring in control group 50% (Figure 3).

The difference in the sex ratio between the first group mothers (Ca, Mg) with control group were statistically significant (Table 2).

The Total No of offspring in Figure 2 ,the first group mothers (Ca, Mg) 70 was equal to total no of offspring the control group 70 in Figure 4.

Table 1:

Estimated Minerals Requirements

Mouse Minerals** (g/Kg)	Amount diet(Kg)	Human *(mg-ug/day)
Calcium	5.0	1000
Chloride	0.5	750
Magnesium	0.5	2-5
Phosphorus	3.0	700
Sodium	0.5	500
Potassium	2.0	2000
Iron	35.0	8
Manganese	10.0	2-5
Zinc	150.0	10-12
Iodine	150.0	150-150
Molybdenum	150.0	75-250(ug)

***adapted from Nutrient Requirements of Nonhuman Primates.*

** Adapted from Lanus Micronutrient information Center, Oregon State Unit.*

Table 2

Sex ratio in different groups of rabbits

Group	Total no of offspring	No. of male offspring	% age of male offspring	No. of female offspring	% age female offspring	Sex ratio
Ca, Mg	70	25	35.71	45	64.29	1.8
Non Ca, Mg	70	35	50	35	50	1

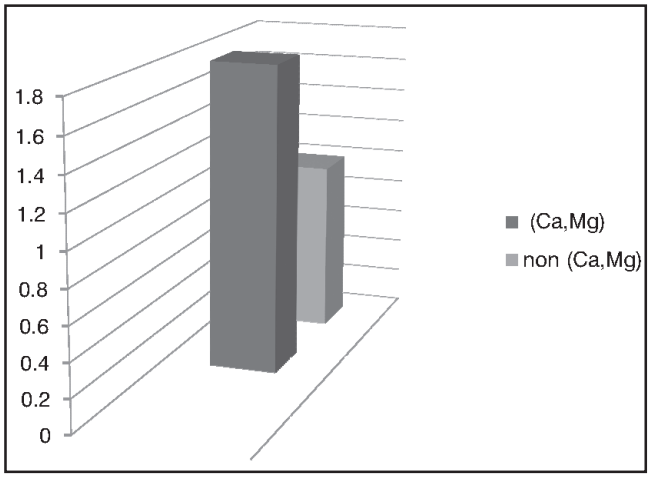

Fig. 1 : Sex ratio of male to female in different groups of rabbits

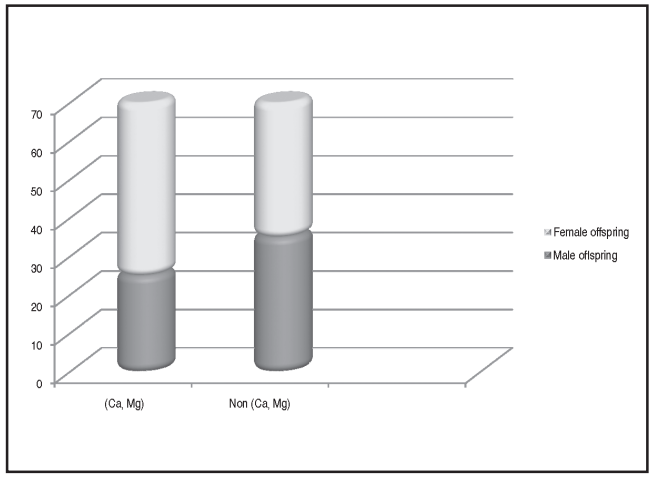

Fig. 2: Number of Male and female in different groups rabbits

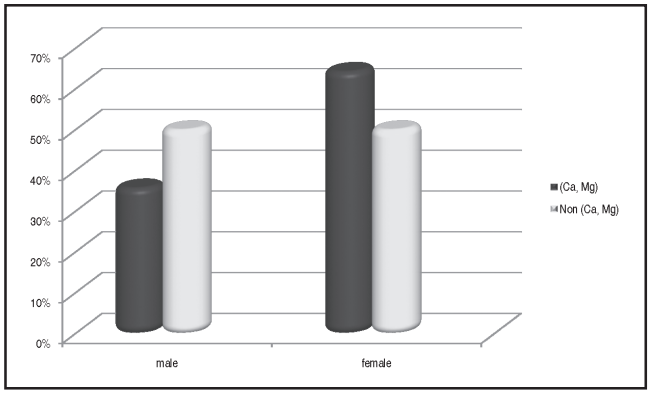

Fig. 3: Percentage of offspring sex in different groups of rabbits

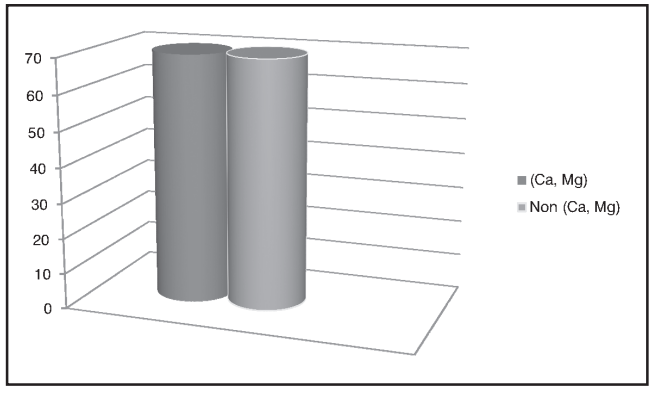

Fig. 4: Number of offspring in different groups of rabbits

The first medical technique that can be used to select for sex is that of pre-natal diagnosis [PND] and abortion. To utilize this method, the pregnant woman must undergo some sort of prenatal testing, such as an amniocentesis, chorionic villus sampling or an ultrasound, which will allow the doctor to determine the sex of the child, among other things. Once the woman has the information about the child's sex, she Nan obtains an abortion if the fetus is not of the desired sex. The use of PND and abortion in order to select for sex sounds extreme, and indeed, as Edgar Dahl points out, it is not common for Westerners to utilize such a technique. For example, a follow-up study of 578 patients having prenatal diagnosis at one Melbourne centre found that none of the women had a termination because of the sex of the fetus .Going through the traumatizing experience of an abortion is usually seen as too high a price to pay for a child of a particular sex[18].

The second medical technique that can be used to select for sex is that of pre-implantation genetic diagnosis [PGD] with *in vitro* fertilization [IVF]. With this technique, the embryos are screened for sex prior to being implanted into the woman's uterus, thereby eliminating the need to later decide to terminate a pregnancy. PGD and IVF, however, are very invasive and potentially physically harmful, requiring the woman to go through at least one IVF cycle, which includes taking potent drugs to induce super-ovulation, extraction, fertilization and then testing and subsequent implantation of the embryos. Given the expense of IVF treatment cycles (according to IVF Canada in 2005 it cost $5,500 for one cycle of IVF, not including drugs[15].and, according to the same source, the drugs themselves Nan cost approximately $3,000 for one cycle[19], it would be highly unlikely that it would be used as a technique for sex selection alone. More likely, it could be used as a sex selection technique for those who are already undergoing IVF for other medical reasons.

The last medical technique that can be used to select for sex is sperm sorting. New technologies allow sperm to be sorted into those carrying X or Y chromosomes with varying degrees of accuracy. To date, the most successful way in which to sort sperm is flow cytometry, which has been branded as the Micro Sort technique [20]. Sex selection using flow cytometry results from distinguishing between the identifiable differences between the X and Y chromosomes, as the X chromosome is larger than the Y. The sorted sperm is then used to artificially inseminate the

woman. Studies have shown that the Micro Sort technique is more effective in selecting for girls, a success rate of 91%, than for boys, with a success rate of only 76%.12 Sperm sorting appears, then, to be the least invasive and least expensive (at about $2,300US per cycle13) method of selecting for sex.

There are also methods which use different food combinations and especial diets to maximum the chance of having a baby with specific sex. The old believe is that eating salty, savory foods leads to delivering a male and sodium rich foods to a female. Some believes that the ratios of the minerals sodium, potassium are important in determination of baby gender. It was shown that pregnant female house mice maintained on a consistent low-food diet give birth to a lower proportion of males than do control females fed ad labium[21].

Conclusion

Today one of good known methods on sex constitution is the preconception diet method. This method claims 80% accuracy and the theory is that by altering your diet to include and exclude certain food, the condition in the reproductive tract will be directly affected; increasing the odds of conceiving a particular sex it is also recommended that both mother and father go on the diet. This is also consistent with the oriental philosophy that everything has a yin or yang quality and the foods supplied in the female diet, female and acid are all yin. The female diet is high in sodium but low in salt and potassium, containing acid forming foods. The diets nutritional content is questionable and contains multiple warnings. The diet may influence the conditions within the reproductive tract and the outer barrier surrounding the ovum. Enabling only one of the two types of sperm to penetrate the depending on which diet is adhered to. Langendon and Proctor first published 'the preconception Gender Diet `based on results reported [22]. The theory is that by altering your diet to include and exclude certain foods, the conditions in the reproductive tract will be directly affected, increasing the odds of conceiving a particular sex. This method under scrutiny claims of 80% accuracy based on one clinical trial of only 260 women, the results were published in the international journal of Gynecology and Obstetrics in 1980. The female diet is high in sodium but low in salt and potassium, aontaining acid forming foods. The diets nutritional content is questionable and contains multiple warnings.

It is recommended to seek the advice of medical practitioner before going on such a restrictive diet, and stay on the diet for no longer than three months. The diet may influence the condition of the cervical mucus and within the reproductive tract and follicular fluid. Enabling only one of the two types of sperm to penetrate the egg depending on which diet is adhered to. The aim of this study was to elevate relationship between minerals and sex ratio in rabbits.

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Synthesis and Biological Evaluation of Some 1-[(2-methyl-1*h*-indol-3-yl) Carbonyl]-3-substituted Phenyl-1*h*-pyrazole-4-carbaldehyde Derivatives

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ABSTRACT

The present investigation is concerned with synthesis, characterization and antimicrobial screening of 1-[(2-methyl-1*H*-indol-3-yl) carbonyl]-3-substituted phenyl-1*H*-pyrazole-4-carbaldehyde derivatives. When one biologically active molecule is linked to another, the resultant molecule generally has increased potency. Hence two pharmacophores, i.e. pyrazole ring and indole moiety are fused to obtain highly potent, more specific and less toxic agent. In the present study , synthesis of title compounds 1-[(2-methyl-1*H*-indol-3-yl)carbonyl] -3-substituted phenyl-1*H*-pyrazole-4-carbaldehyde derivatives(VIa-e) by using Vilsmeier-Haack reagent (DMF/$POCl_3$). The facial synthesis of 1-[(2-methyl-1*H*-indol-3-yl) carbonyl] -3-substituted phenyl-1*H*-pyrazole-4-carbaldehyde derivatives (VIa-e) has been achieved by the reaction of phenyl hydrazine (I) with a mixture of ethylacetoacetate(II) and glacial acetic acid to synthesize ethyl-2-indole-3-carboxylate(III) which on reaction with hydrazine hydrate in ethanol to form 2-methyl-1H-indole-3-carbohydrazide (IV) which on condensation with different acetophenones in methanol in the presence of glacial acetic acid affords hydrazones (Va-e).The hydrazones (Va-e) on treatment with Vilsmeier-Haack reagent furnished 1-[(2-methyl -1*H*-indol-3-yl) carbonyl] -3- substituted phenyl -1*H*- pyrazole -4- carbaldehyde derivatives (VIa-e).

Key words: Indole, pyrazole, antimicrobial activity, Vilsmeier-Haack reagent.

Introduction

The indole moiety is probably the most widely spread nitrogen heterocycle in nature. The biological importance of iodole and pyrazole made them extremely attractive and rewarding research targets and has motivated countless researchers to study their synthesis and pharmacological properties. Indole is the most beneficial heterocyclic nucleus which has gained prominence in medicinal chemistry due to its diverse biological activities such as Antimicrobial [1-3], Anti-inflammatory [4-5], anticonvulsant [6], tranquilizer [7], CNS Depressant Activity [8] and pyrazole is also found to possess pharmacological activities like antimicrobial [9-10], antitumor [11], analgesic [12]. It is therefore thought worthwhile to synthesize some new indole derivatives by incorporating pyrazole moiety in a single molecular frame work with the hope to posses better antimicrobial activity. The required starting compound is ethyl-2-indole-3-carboxylate (III) was prepared by the Fischer- Indole synthesis. These compounds underwent condensation with hydrazine hydrate in ethanol to furnish the Indole-3-carboxy hydrazide (IV).The reaction of hydrazides with different acetophenones in methanol in the presence of acetic acid to form (V). These compounds treated with Vilsmeier-Haack reagent (DMF/$POCl_3$) to form title compounds (VIa-e).

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Experimental

Material & Methods

Melting points of all synthesized compounds were determined in open capillary tubes on an electro thermal apparatus and are uncorrected. The progress of the reaction and purity of the compounds was checked by TLC on silica

gel coated aluminium plates (Merck) as adsorbent and UV light as visualizing agent. UV Spectra were obtained on ELICO SL 244 UV Double Beam Spectrophotometer.IR spectra (KBr ν_{max} cm^{-1}) were recorded on a BRUKER FTIR spectrophotometer in the range of 4000-400 cm^{-1} .^{1}HNMR spectra were recorded on a INOVA (400 MHz) NMR spectrometer using $CDCl_3$ as solvent and TMS as an internal standard (chemical shifts in δ ppm).Mass spectra were recorded on a VG Autospec MS using ESI mode positive ion trap detector. The drugs and fine chemicals were purchased from Sigma-Aldrich, India. All other chemicals and solvents were obtained from local firms (India) and were of highest pure and analytical grade.

SYNTHESIS

Synthesis of Ethyl-2-methyl-1H-indole-3-carboxylate [13] (III).

In a flat bottom flask (250 ml) fitted with a dropping funnel, a sealed stirrer unit and reflux condenser, a mixture of ethylacetoacetate (II) (6.3 ml; 0.05 mol) and glacial acetic acid (3ml, 0.05mol) was placed in the flat bottom flask and heated under reflux with stirring. Phenyl hydrazine (I) (5 ml; 0.05mol) was slowly added during first 1hr stirring is continued for further 1hr. The reaction mixture was poured into a 50ml beaker and stirred vigorously while it solidifies. Then sufficient water is added and filtered. The crude product is dried, and recrystallized from ethanol.

Synthesis of 2-methyl-1H-indole-3-carbohydrazide (IV).

Ethanolic solution of Ethyl-2-methyl-1H-indole-3-carboxylate (III) (10gm 0.05mol) was refluxed with hydrazine hydrate (2.5 gm; 0.05 mol) for 3 hr at 70°C.The reaction mixture was allowed to cool and poured over crushed ice. The solid thus obtained was filtered and dried. The crude product thus obtained was recrystallized from ethanol.

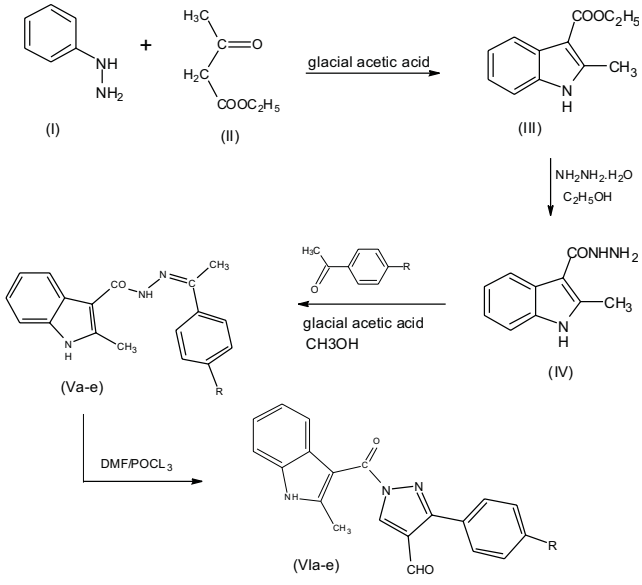
General procedure of synthesis of 2-methyl-*N*'-[(1*E*)-1-phenylethylidene]-1 *H*- indole-3-carbohydrazide (Va-e).

A mixture of 2-methyl-1H-indole-3-carbohydrazide (IV) (9.45 gm; 0.05mol) and acetophenone derivatives (5.85 ml, 0.05 mol) in 25ml of methanol containing 3-4 drops of glacial acetic acid was refluxed for 2 hr at 70°C.The reaction mixture was allowed to cool to room temperature. The reaction mixture is poured into cool water and stirred well and kept aside for overnight in refrigerator. The crude product was recrystallized by mixture of solvents acetone and water.

General procedure of synthesis of Title compounds Synthesis of 1-[(2-methyl-1*H*-indol-3-yl) carbonyl]-3-phenyl-1*H*-pyrazole-4-carbald- ehyde (VIa-e)

The compounds 2-methyl-*N*'-[(1*E*)-1-phenylethylidene]-1*H*-indole-3-carbohydrazide (VIa-e) (14.5 gm;0.05 mol) was added to the mixture of Vilsmeier-Haack reagent, prepared by drop wise addition of phosphorus oxychloride (1.5 ml; 0.05 mol) to an ice cold solution of N,N dimethyl formamide(20ml). The reaction mixture was refluxed for 4hrs at room temperature by using magnetic stirrer. Then the reaction mixture was poured into ice cold water and neutralized with sodium bicarbonate solution. The product obtained was filtered, washed with water and recrystallized from ethanol (Scheme-1). The physical data of the synthesized compounds are given in the table1.

Scheme 1



Results and Discussion

In the present study , synthesis of title compounds 1-[(2-methyl-1*H*-indol-3-yl) carbonyl] -3-substituted phenyl-1*H*-pyrazole-4-carbaldehyde derivatives (VIa-e) by using Vilsmeier-Haack reagent (DMF/$POCl_3$).As a starting compound ethyl-2-indole-3-carboxylate (III) used to synthesise Schiff bases. The structure of compounds was characterized by IR, ^{1}H NMR and Mass spectral data.

Spectral data of 1-[(2-methyl-1*H*-indol-3-yl) carbonyl]-3-(4-methylphenyl)-1*H*-pyrazole-4-carbaldehyde (VIa)

IR (KBr):3415 cm^{-1} (indole NH), 3150 cm^{-1} (C-H), 1620 cm^{-1} (C=O), 1500 cm^{-1} (C=N),1120 cm^{-1}(C-O).^{1}HNMR(ppm):δ10.1(s, 1H, indole-NH), δ9.6(s, 1H, CHO), δ7.5(s, 1H, pyrazole-CH), δ7.0-7.4(m, 8H, ArH), δ2.3 (s, 3H, CH_3), δ2.4(s, 3H, CH_3), MS-m/z 343(M+).

Spectral data of 3-(4-ethylphenyl)-1-[(2-methyl-1*H*-indol-3-yl) carbonyl]-1*H*-pyrazole-4-carbaldehyde (VIb)

IR(KBr):3403cm^{-1}(indole-NH), 3046 cm^{-1}(C-H), 1665 cm^{-1} (C=O), 1580 cm^{-1} (C=N), 1254 cm^{-1} (CO), ^{1}H NMR (ppm): δ10.2(s,1H, indole-NH), δ9.5 (s, 1H, CHO), δ7.5 (s, 1H, pyrazole-CH), δ6.5-8.1(m, 8H, ArH), δ2.3(s, 3H, CH3), δ2.60(q, 1H, CH), δ1.24(d, 3H, CH_3), M.S(m/z): MS-m/z 357(M+).

Table-1

Physical Data of The Synthesized Compounds

Compound	R	M.F	M.W	M.P (°C)	Yield (%)
VI a	$-CH_3$	$C_{21}H_{17}N_3O_2$	343	83-85	64
VI b	$-CH_3CH_2$	$C_{22}H_{19}N_3O_2$	357	68-70	55
VI c	-F	$C_{20}H_{14}FN_3O_2$	347	72-74	52
VI d	-Cl	$C_{20}H_{14}ClN_3O_2$	364	64-68	68
VI e	$-OCH_3$	$C_{21}H_{17}N_3O_3$	359	80-82	62

Spectral data of 3-(4-fluorophenyl)-1-[(2-methyl-1*H*-indol-3-yl) carbonyl]-1*H*-pyrazole-4-carbaldehyde (VIc)

IR(KBr):3420 cm^{-1} (indole-NH), 3040 cm^{-1} (C-H), 1676 cm^{-1} (C=O), 1599 cm^{-1} (C=N), 574 cm^{-1} (C-F), ^{1}HNMR(ppm): δ 10.6 (s, 1H, indole-NH), δ 9.2 (s, 1H, CHO), δ7.8 (s, 1H, pyrazole-CH), δ 6.5-7.9(m, 8H, ArH), δ2.5(s, 3H, CH3), MS-m/z 347(M+).

Spectral data of 3-(4-chlorophenyl)-1-[(2-methyl-1*H*-indol-3-yl) carbonyl]-1*H*-pyrazole-4-carbaldehyde (VId)

IR (KBr):3405cm^{-1}(indole-NH), 2924 cm^{-1} (C-H), 1702 cm^{-1} (C=O),1594 cm^{-1} (C=N),1252 cm^{-1} (C-O), ^{1}H NMR (ppm): δ10.3(s, 1H, indole-NH), δ9.2(s, 1H, CHO), δ8.3(s, 1H, pyrazole-CH), δ6.5-8.8(m, 8H, ArH), δ 2.3(s, 3H, CH3), MS-m/z 364(M+).

Spectral data 3-(4-methoxyphenyl)-1-[(2-methyl-1*H*-indol-3-yl) carbonyl]-1*H*-pyrazole-4-carbaldehyde (VIe)

IR(KBr):3408cm^{-1}(indoleNH), 3055cm^{-1} (C-H), 1660cm^{-1} (C=N), 1273cm^{-1} (C-O), ^{1}H NMR (ppm):δ11.2(s, 1H, indole-NH), δ9.8(s, 1H, CHO), δ8.7(s, 1H, pyrazole-CH), δ 6.7-8.0(m, 8H, ArH), δ 3.8(s, 3H,OCH_3), MS-m/z 359(M+)

Antimicrobial Activity

The antimicrobial activity of the synthesized compounds was determined by cup-plate method **[14]**. The organisms selected for antibacterial activity were two gram-positive bacteria *viz*., *B.Substilis, S.aureus* and two gram-negative bacteria *viz*., *E.coli, P.aeruginosia.* Similarly the antifungal activity was carried out by using *viz*., *Aspergillus niger* and *Candida albicans*. The concentration of sample compounds was 100µg/mL. Norfloxacin and Ketoconazole were used as standard drugs for antibacterial and antifungal activity respectively. Control test with solvents were performed for every assay but showed no inhibition of the microbial growth. All the compounds synthesized have shown potent to weak antibacterial activity. Compounds VIc and VId shows good activity against S.aureus and E.coli when compared with standard. Compounds VIb and VIa and VIe showed moderate antibacterial activity when compared to standard. The observed data on the antimicrobial activity of the compounds and control drugs are given in table-2

Conclusion

The two moieties i.e. indole and substituted pyrazole moieties fused and screened for possible antimicrobial

Table-2

ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY (VIa-e)

Compound	Antibacterial activity				Antifungal activity	
	S.aureus	B.subtilis	E.coli	P.aeruginosa	A.niger	C.albicans
VI a	18	19	14	17	17	13
VI b	15	16	16	20	16	12
VI c	24	22	21	20	14	17
VI d	20	21	17	14	24	21
VI e	18	18	13	10	11	10
Ciprofloxacin	**25**	**23**	**29**	**25**	—-	—-
Ketconazole	—-	—-	—-	—-	**29**	**26**

(Zone of inhibition measured in mm)

studies, they showed mild to moderate antimicrobial activity, but none of the derivatives showed comparatively good antimicrobial activity in comparison with the standard drugs. The above results establish the fact that pyrazole substituted derivatives can be a potential source for exploitation in search of new generation of antibiotics. It may be worthwhile to explore the possibility in this area by fusing other heterocyclic moieties and increase the potency of the synthesized compounds.

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Chitosan: Naturally Occurring Biopolymer for Defluroidation of Water

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ABSTRACT

Present investigation deals with removal of fluoride from drinking water using low cost naturally available biopolymer adsorbent chitosan. The effects of various physico-chemical parameters such as pH, adsorbent dose, initial fluoride concentration and the presence of interfering co-ions on adsorption of fluoride were studied. It was observed that the uptake of fluoride was higher at self pH (pH≅5). The equilibrium adsorption data were fitted well for both Langmuir isotherm model and Freundlich isotherm model. It was observed that co-ions such as sodium nitrate, sodium chloride, sodium sulfate, ferrous sulfate and copper sulfate have positive effect on the uptake of fluoride from drinking water while sodium carbonate and bicarbonate has a negative effect on adsorption of fluoride. The comparison of uptake of fluoride in distilled water and field water shows slightly higher uptake of fluoride in distilled water. This may be because of latter contains different types co-ions and higher pH of the field water.

Keywords: Removal of fluoride; chitosan; biopolymer; defluoridation.

Introduction

Fluoride is an essential constituent for both humans and animals depending on its concentration in drinking water. The presence of fluoride in drinking water, within permissible limits is beneficial for the production and maintenance of healthy bones and teeth, while excessive intake of fluoride causes dental or skeletal fluorosis [1-2]. Higher level of fluoride in groundwater is a world-wide problem, which includes various countries from Africa and Asia as well as USA [3]. Fluoride is one of the most abundant constituent occurring in groundwater in India and creates a major problem in safe drinking water supply. The concentration of fluoride in drinking water is as high as 30 mg l^{-1} in some places. Excess fluoride in drinking water is prevalent in 150 districts of 17 States in India [4]. According to the Department of Drinking Water Supply under Ministry of Rural Development, India, rural drinking water supply is mainly dependent on groundwater (85%). Hence, it becomes necessary to bring down the fluoride concentration within permissible limit of 1.5 mg l^{-1} according to Indian Standards. The limit varies among countries and the age of people exposed. World Health Organization (WHO) has set a limit range between 0.5 and 1 mg l^{-1} [5]. According to US standard it is between 0.6 and 0.9 mg l^{-1}.

Chitosan is an interesting and abundant polysaccharide, found in a wide range of organisms including bacteria and fungi, but commercially most commonly extracted from shellfish processing waste. The structure of chitosan is shown in Fig.1a. It is generally considered to be, the most abundant biopolymer in the ecosphere after cellulose which it resembles structurally. A number of publications have been reported mainly on alumina [4,8-10], calcium [6,11-13], clays [14-19] and biopolymer [20-22] however, to the best of our knowledge chitosan, has not been studied so far towards its properties for defluoridation of water. The present investigation deals

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with removal of fluoride from drinking water by using low cost materials such as chitosan. The effects of the various physico-chemical parameters such as pH, adsorbent dose, initial fluoride concentration and presence of co-ions on removal of fluoride were investigated. Ground water samples collected from SPSR Nellore district of Andhra Pradesh, India, were also used for fluoride removal studies. The detailed characteristics of field water are given in Table 1.

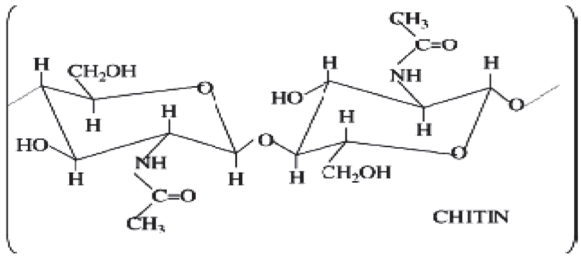

Fig. 1: Structure of chitosan unit

Experimental

Materials and Methods

Chitosan was purchased from Chemchito, Chennai. A stock solution of fluoride was prepared by dissolving sodium fluoride in distilled water and working fluoride solution of 5mg l^{-1} was prepared from stock fluoride solution by appropriate dilution. Batch adsorption experiments were conducted to investigate the effect of various parameters. Fluoride was estimated using Ion selective electrode. The specific amount of fluoride adsorbed was calculated from:

$$q_e = (C_o - C_e)\, x \frac{V}{W} \quad (1)$$

Where q_e is the adsorbate loading (mg g^{-1}) in the solid at equilibrium; C_0, C_e are initial and equilibrium concentrations of fluoride (mg l^{-1}), respectively; V is volume of the aqueous solution and W is the mass (g) of adsorbent used in the experiments.

The effect of solution pH on fluoride removal was studied by adjusting the pH of the solution by using 0.1N HCl or 0.1N NaOH.

Result and Discussion:

Characterization:

XRD of chitosan and Iron loaded chitosan flakes are shown in Fig. 2a. These spectra show that chitosan exhibits a narrow high peak, at 2è = 20°, and a wide lower peak at 2è = 10°, which is a typical pattern of a chitinous material. The main IR bands of chitosan spectrum were retained on loading of iron in chitosan, especially those related to N-H vibrations of $–NHCOCH_3$ group, located at 2886, 1440 and 1369 cm^{-1} and C-O virabation at 1159 and 1014 cm^{-1} (see Fig. 2b). Surface morphology of Chitosan was studied using SEM (Fig. 1c). Chitosan have dense, firm and a rough surface without porosity

Fluoride removal using chitosan:

Chitosan can be deacetylated using NaOH where acetamide ($–NHCOCH_3$) group of chitosan, Chitosan is converted to amine ($–NH_2$) group. Fluoride removal capacity of chitosan and deacetylated chitosan has been compared and the results are given in Fig.3. It is observed that with increase in % deacetylation of chitosan fluoride removal decreases which may be due to decrease in surface area with increase in % deacetylation of chitosan.

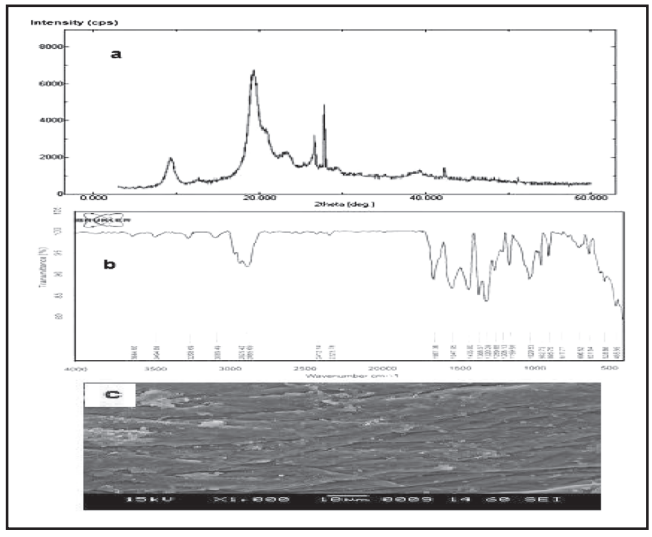

Fig. 2: Characterization a) XRD pattern, b) FTIR spectrum, c) SEM image of Chitosan

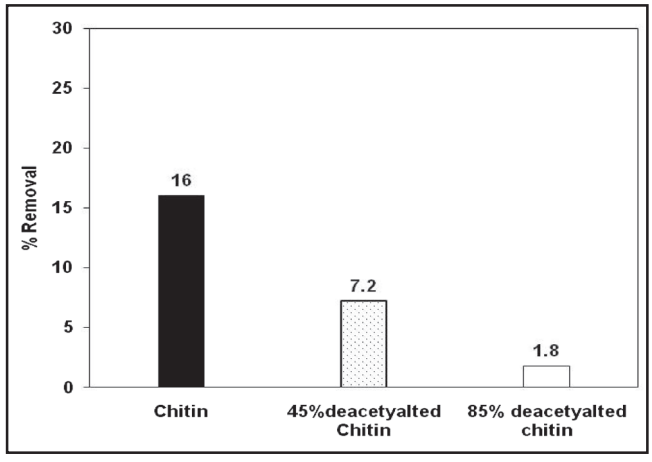

Fig. 3: Fluoride removal using chitin and deacetylated chitosan

Effect of presence of co-anions

The effect of presence of co-ions on the removal of fluoride is shown in Fig. 6. It was observed that sodium chloride, sodium sulfate, sodium nitrate, sodium bicarbonate, ferrous sulfate and copper sulfate ions shows positive effect on removal of fluoride. It was also found that sodium bicarbonate has slightly negative effect on removal of

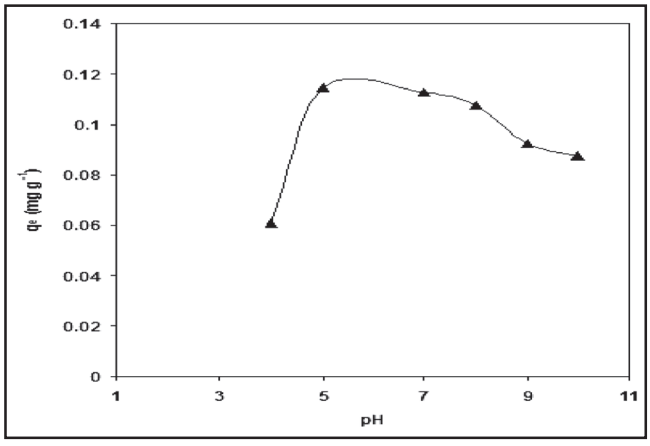

Fig. 4: Effect of pH

fluoride. This may be because of the change in pH as well as the competing effect of this co-ion. The pH of the fluoride solution were 7.07, 6.58 7.06, 3.28, 4.17, 9.07, 10.84, respectively for sodium chloride, sodium sulfate, sodium nitrate, ferrous sulfate, copper sulfate, sodium bicarbonate sodium and sodium carbonate while the pH of the fluoride solution was 7.29 without addition of co-ions. This indicates that addition of some of co-ions resulted in decrease in pH of fluoride solution except sodium carbonate and bicarbonate. From our experiments on effect of pH it was observed that the adsorption of fluoride was higher in the pH range of 5-7. Overall it was observed that the presence of cations and many of the anions enhance the uptake of fluoride from aqueous solution indicating fluoride specific sorption behavior.

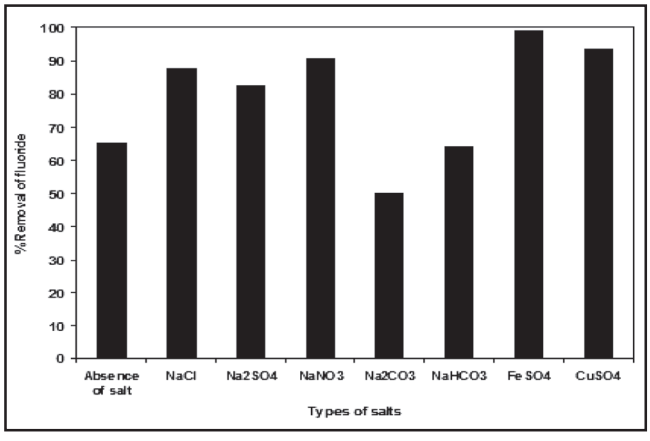

Fig. 6: Effect of the presence of co-existing ions

Equilibrium isotherm studies

Equilibrium studies were carried out to determine the maximum fluoride removal capacity and equilibrium constant on chitosan. It was observed that adsorption capacity reaches an equilibrium value beyond which there was negligible change in the residual fluoride concentration. The distribution of fluoride between the liquid phase and the solid phase is a measure of the position of equilibrium in the adsorption process and can be expressed by the Freundlich and Langmuir equations [19, 20]. These two models are widely used, the former being purely empirical and the latter assumes that maximum adsorption occurs when the surface is covered by the adsorbate. The value of K_F is 0.195 mg g^{-1} and n is 0.849 for Freundlich isotherm. The values of Langmuir parameters, q_{max} and K are 0.275 mg g^{-1} and 0.107 l mg^{-1}, respectively. Value of $r < 1$ represents favorable adsorption. The r-value for the initial concentration of 5 mg l^{1} was found to be 0.66. The value obtained shows that the system is favorable for adsorption of fluoride.

Comparison of fluoride uptake in distilled water and field water

The uptake of fluoride from field water samples (Marripadu) collected from Andhra Pradesh, India by using chitosan is shown in Fig. 7. This Figure also illustrates removal of fluoride from distilled water. It was observed that the percentage removal of fluoride in distilled water is slightly higher as compared to field water sample. The detailed physico-chemical characteristics of field water samples before treatment are given in Table 1. Table 1 show that field water has TDS of = 477 mg l^{-1} which is indicative of presence of other ions in addition to fluoride. This could be due to two reasons i) pH of the field water samples is alkaline and ii) it contains different types of co-ions. These ions compete with adsorption of fluoride on chitin and reduce the adsorption of fluoride. In section 3.4 it has been discussed that in the alkaline pH uptake of fluoride is low as compared to acidic pH. Therefore, overall removal of fluoride in field water is low as compared to distilled water.

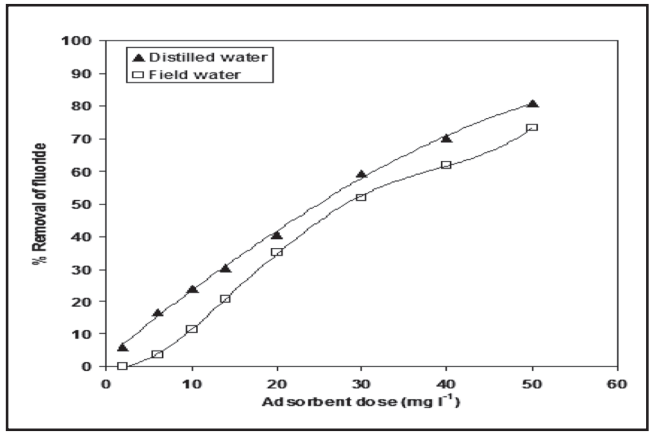

Fig. 7: Comparison of adsorption of fluoride in distilled water and field water

Conclusion

A naturally occurring bio-sorbent such as chitosan is found to be a suitable adsorbent for removal of fluoride from drinking water. The adsorption of fluoride on the surface of the adsorbent is observed to dependent mainly on the pH of the solution, initial concentration of fluoride and presence of co-anions. It was observed that in the presence of cations and most of anions (except sodium

carbonate and bicarbonate) have a positive effect on the adsorption of fluoride. The adsorption of fluoride at acidic pH (pH =5) was high as compared to alkaline pH. The percentage removal of fluoride in distilled water was higher than field water; this may be because latter contains different types of ions and is having alkaline pH.

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