Indian Journal of Advances in Chemical Science

Article

Biological Activity and Variation of Toxicity of Probenecid in Diverse Solvent Systems Probed by Physicochemical Investigation

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ABSTRACT

Probenecid has an extended and storied history in medicine that has been characterized by unexpected usefulness and a very benign toxicity profile. The solubility of probenecid (PB) in water (H_2O), ethanol (EtOH), ethoxyethanol, ethyl acetate (EtOAc), and ({ethyl acetate (EtOAc) + ethanol (EtOH)}, {ethyl acetate + ethoxy ethanol} binary co-solvent mixtures) was determined at three different temperatures from T = 298.15 K to T = 308.15 K was determined consecutively to open further fields in the clinical treatments.

Key words: Ion-solvent interaction, Cytotoxicity, Probenecid, Ethyl acetate-ethoxy-ethanol, Fluorescence

1. INTRODUCTION

Probenecid (PB) also known as Probalan, molar mass 285.36 g/mol, IUPAC name: 4-(dipropylsulfamoyl) benzoic acid, CAS number: 57-66-9 is a commonly employed uricosurics drug, which increases uric acid exclusion in the urine. It is used to prevent gout as well as gouty arthritis. PB also reduces the renal tubular excretion of various other drugs and increases their plasma concentration. To have biological activity, drug molecules should be able to penetrate the lipophilic cell membrane but as the solubility of (PB) is very low, it is difficult to apply (PB) in human serum. Hence, the mechanical elasticity known in the anti-hyperuricemia drug probenecid has been extended into multi-component systems using active solvent sites and studied in the present work.

Drug interactions can be broadly be divided into pharmaceutical interaction which denotes dissolving the drug in diverse solvents. Experimental determination of the solubility of drug molecules in (aqueous + organic cosolvent) systems is desired to design effective purification processes and to formulate suitable pharmaceutical drug delivery systems. PB equilibrium solubility in water at room temperature is low and (PB) is, thus, classified as a very slightly soluble drug. Added cosolvents often provide a convenient means to increase the solubility of the slightly soluble drug molecule. Researchers have measured the solubility of PB in several {(EtOAc) (1) + hydroxyl-co-solvent (2)} mixtures to provide useful information that is needed to develop some pharmaceutical dosage forms and/or to understand the main mechanisms involved in the drug solubilization. For example, the solubility and solution thermodynamics of PB has been studied in {ethyl acetate (EtOAc) (1) + ethanol (EtOH) (2)}, {ethyl acetate(1) + ethoxyethanol(2)}, and {ethyl acetate (EtOAc) (1) + water(H_2O) (2)} […] binary mixtures. The main goals of this research study are the following: (i) To determine the equilibrium solubility of PB in several {ethyl acetate (EtOAc) (1) + ethanol (EtOH) (2)} mixtures at three temperatures from 298.15 to 308.15 K, (ii) to calculate the respective thermodynamic quantities of solution and mixing of this drug in this binary (aqueous + organic cosolvent) system, and (iii) to estimate the respective preferential solvation parameters of SMT as a function of binary solvent composition. In this present work, we attempt to ascertain the formation and nature of interactions with (PB) in diverse solution environment by spectroscopic and physicochemical studies, so that, the drug may be released in a controlled fashion without any chemical and biological modification [1-7].

2. EXPERIMENTAL SECTION

2.1. Source and Purity of Samples

Probenecid of the pure grade was bought from Sigma-Aldrich, and other solvents used were bought from Thomas Baker (Chemicals) Pvt. Ltd. The mass fraction purity of probenecid and other chemicals was taken to be ≥0.99 and ≥0.98, respectively.

2.2. Apparatus and Procedure

The solubility of PB is less in pure water. Thus, all the working solutions have been prepared by taking 1:1 molar ratio of PB in various pure solvents including the cosolvents and also in triply distilled and degassed water maintaining pH of the solution at 7.0. All stock solutions were prepared by mass (weighed by Mettler Toledo AG-285 with uncertainty 0.0003g) at 298.15K.

Infrared spectra were recorded in 8300 FT-IR spectrometer (Shimadzu, Japan) and the particulars about this instrument have been illustrated previously [8].

UV–visible spectra were taken by JASCO V-530 UV/VIS spectrophotometer, with uncertainty in wavelength as ±2 nm. The temperature during the experiment is kept constant using a digital thermostat.

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ISSN NO: 2320-0898 (p); 2320-0928 (e)
DOI: 10.22607/IJACS.2018.604004

Received: 22nd August 2018;
Accepted: 08th December 2018

The fluorescence measurements were done with Quanta Master 40 spectrofluorometer. The output range of the machine was nearly two analog (±10 volts).

The conductance measurements were taken in a Systronic-308 conductivity meter (accuracy ± 0.01) using a dip-type immersion conductivity cell, CD-10, having a cell constant of approximately (0.1± 0.001) cm^{-1}. Measurements were made in a water bath maintained within T = (298.15 ± 0.01) K, and the cell was calibrated by the technique proposed by Lind *et al*. The conductance data were obtained at a frequency of 1 kHz.

Antimicrobial activity assay: Gram-negative *Escherichia coli* and *Aeromonas hydrophila* and Gram-positive *Bacillus cereus* and *Lactobacillus* sp. were considered as the model organisms. The experiments were done following the Agar cup method (Vesterdal, 1946). In brief, each organism was separately inoculated on Mueller-Hinton agar plates following the spread plate technique, and the compounds were dispensed in wells cut in the agar plates. The plates were incubated at 37°C for 24 h. Probenecid (5 mg/ml) was dissolved in three solvents, namely water, ethoxyethanol, and ethyl acetate separately. The experiments were done in three separate sets. Different doses of probenecid ranging from 10 to 500 µg were used to check the antimicrobial property of the compound if any. 100 µg ampicillin (10 mg/ml) and 50 µg tetracyclin (5 mg/ml) were used as positive controls, and the solvents, namely water, ethoxyethanol, and ethyl acetate were used as negative controls. The antimicrobial activity was determined by measuring the diameter of the zones of inhibition formed if any around the agar cups. All the experiments were repeated thrice.

3. RESULTS AND DISCUSSION

3.1. UV-study

The absorption spectra of pure solvents used in work such as H_2O, ethoxyethanol, and ethanol and their mixtures with EtOAc in binary and ternary mixtures with probenecid (PB) are depicted in Figure 1. The absorption spectrums of pure solvents are almost analogous, but in their mixtures, the spectrums are slightly different. Absorbance versus wavelength spectra clearly shows that there is a shifting of λ_{max} value is 380 nm in the mixture of PB and diverse solvent solutions. The shifting of λ_{max} value is greater for the mixture of PB and EtOAc + EtOH than the other studied systems. Thus, these UV-Visible spectra study supports the fact which is discussed later in the section of density, viscosity, and refractive index study [8,9].

3.2. Fluorescence Study

In former studies, absorbance values were observed at 380 nm at 298.15 K for a series of solutions. Here, the excitation was affected at 385 nm and emission was observed at 435 nm. Moreover, the fluorescence experiment was done in the diverse solvent mixture. The system which has less fluorescence intensity will show more interaction (Figure 2). Therefore, according to our observation in the binary solvent mixture of (EtOAc + Ethoxyethanol) superior interactions with PB compared to the mixture of EtOAc + EtOH [2,10].

3.3. Infrared Spectra

FTIR spectra were obtained for PB in pure as well as for cosolvents in various concentrations. Alcohols used in the present study have characteristic IR absorptions associated with both O-H and the C-O stretching vibrations. The spectrum of pure ethanol shows very broad, strong band of the O–H stretch (3391), and the C–O stretches (1102, 1055) are observed. Spectra of [EtOAc + EtOH] with PB is shifted to lower frequency; they may be due to (a) the free C=O group of ethyl acetate, (b) 1:1 bonding interaction $R_2C{=}O{---}H{-}O{-}C_2H_5$, and (c) 1:2 H bonding as in Figure 3.

The spectrum of pure ethoxyethanol is shown at 1450, 1150, and 2900 cm^{-1} for both the C-O, O-H stretches were observed. Spectra of (EtOAc + ethoxy ethanol) with PB are not shifted to lower frequency region but to higher frequency region showing blue shift, thereby showing strong interaction as shown in Figure 4. The shift in peak position usually means the electron distribution in the molecular bond has changed, i.e., change in vibrational frequency which may be either due to the change in bond strength or change in reduced mass, can occur in interacting systems [8,11].

3.4. Conductivity

The Onsager equation for entirely dissociated electrolytes is

$$\Lambda=\Lambda_0-(A+B\Lambda_0)\,C^{1/2} \quad (1)$$

Where A and B are independent of the concentration of used electrolytes. It satisfactorily accounts from a change in equivalent conductivities with concentration. Correct evaluation of Λ_0 can be made by extrapolating to zero concentration of the line obtained by plotting Λ and $C^{1/2}$. However, the method of extrapolation has been reported to be unreliable in case of a number of electrolytes concerning incomplete dissociation or ion association. The extended Onsager's equation tabulated dissociation constants

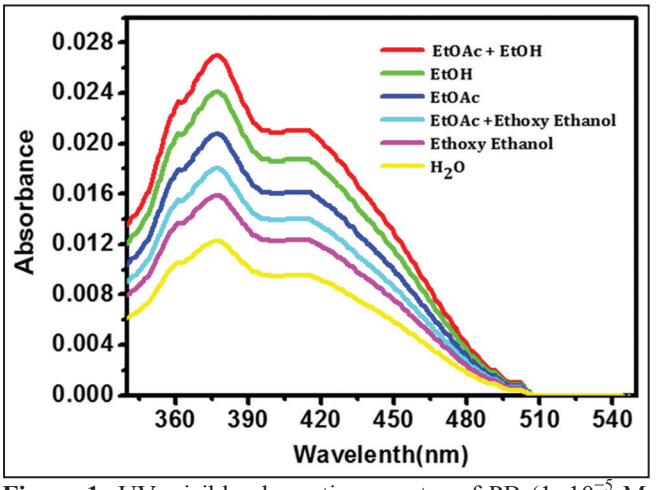


Figure 1: UV–visible absorption spectra of PB (1×10^{-5} M in diverse solvents) at 298.15 K.

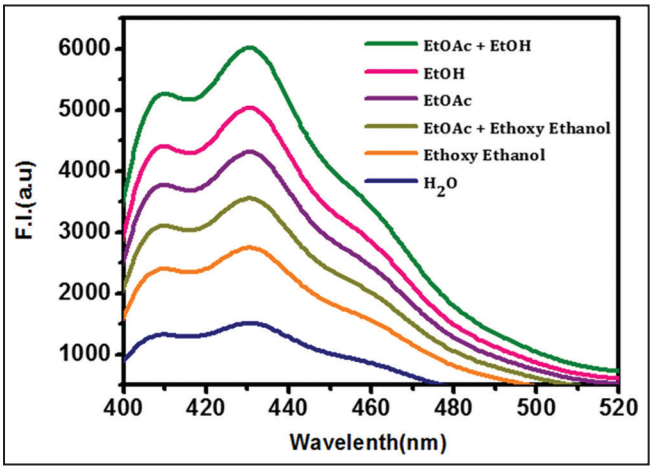


Figure 2: Fluorescence emission spectra of diverse solvents in the presence of 0.1–1.0 mM of PB (λex = 275 nm, slit = 5/5).

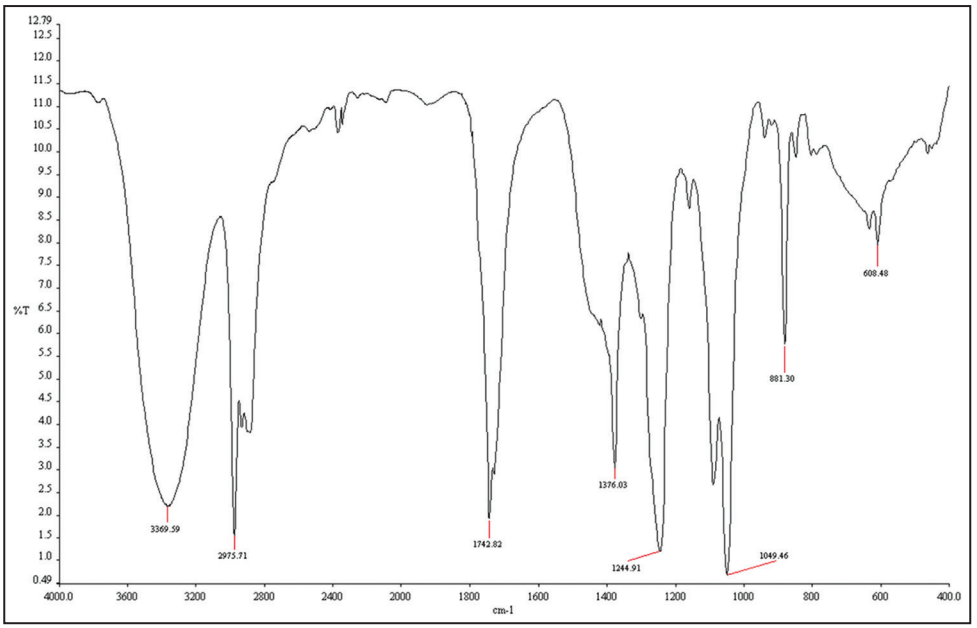


Figure 3: Infrared spectra for (EtOAc + EtOH + PB) solution system.

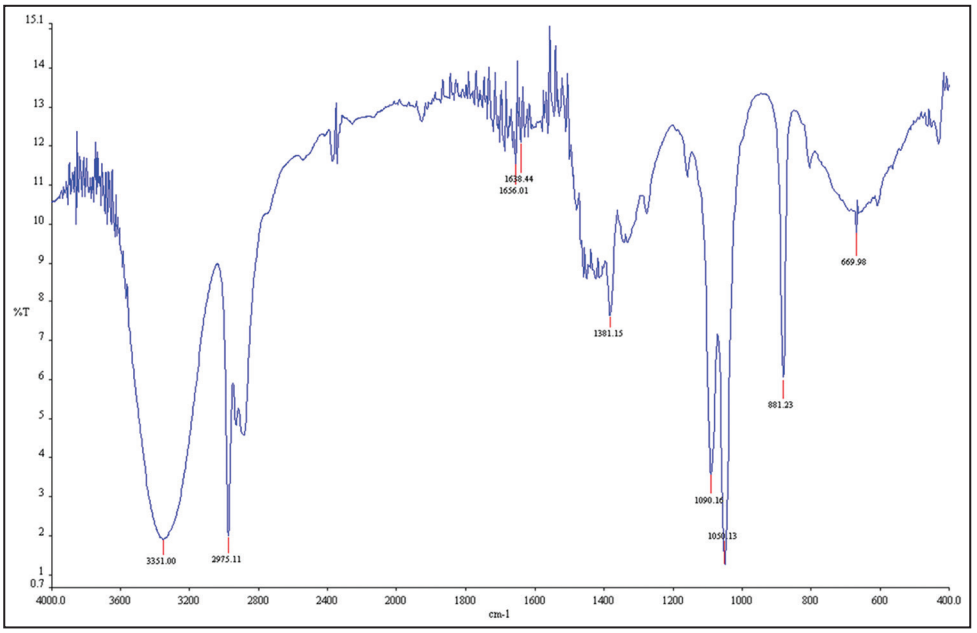


Figure 4: Infrared spectra for (EtOAc + ethoxy ethanol + PB) solution system.

Table 1: The concentrations (c) and molar conductance (Λ) of PB in binary solvent mixture EtOH+EtOAc at 298.15 K, 303.15K, 308.15K.

c * 10^4/mol/dm^{-3}	At 298.15K	At 303.15K	At 308.15K
	Λ/S cm^2/mol	Λ/S cm^2/mol	Λ/S cm^2/mol
0.3525	11.071	11.086	11.092
0.3684	10.880	10.965	10.987
0.4534	10.653	10.957	10.963

Table 2: The concentrations (c) and molar conductance (Λ) of PB in binary solvent mixture Ethoxyethanol+EtOAc at 298.15 K, 303.15K, 308.15K.

c * 10^4/mol/dm^{-3}	At 298.15K	At 303.15K	At 308.15K
	Λ/S cm^2/mol	Λ/S cm^2/mol	Λ/S cm^2/mol
0.3829	0.215	0.234	0.256
0.4284	0.211	0.223	0.253
0.4534	0.196	0.218	0.234

of a variety of salts, especially higher valency type. Electrolytic conductivities have been used to study ion-solvent interaction and solvents of various cations and anions in aqueous and non-aqueous solution. The equivalent conductance of PB investigated by weight percentage of water, ethoxyethanol, (ethyl acetate + ethoxyethanol), ethyl acetate (EtOAc), ethanol, ethyl acetate (EtOAc) + ethanol mixtures (0.01M) at 298.15K, 303.15K, and 308.15K found to be almost linear with $C^{1/2}$. The conductance

Table 3: Limiting ionic conductance (Λ_0) and the corresponding concentration (c) of the studied drug (PB) in different solvents at T=298.15 K, 303.15K, 308.15K.

EtOH+EtOAc		Ethoxyethanol+EtOAc	
T/K	$\Lambda_0 \pm 10^4$/ S m^2/mol	T/K	$\Lambda_0 \pm 10^4$/S m^2/mol
298.15K	11.343	298.15K	0.226
303.15K	12.212	303.15K	0.239
308.15K	12.731	308.15K	0.240

of an electrolyte solution increases with rise in temperature due to enhancement in the extent of ionization (Tables 1 and 2). From the equivalent conductance measurements, we can tell that in (ethoxyethanol + EtOAc) the value decreases due to the more interionic attraction with PB, which has also been proved by spectroscopic techniques in this work. The limiting equivalent conductance of PB leads to the conclusion that with the increase in temperature the ionic size becomes much more effective to be entirely associated in the solute-solvent interaction (Table 3) [12,13].

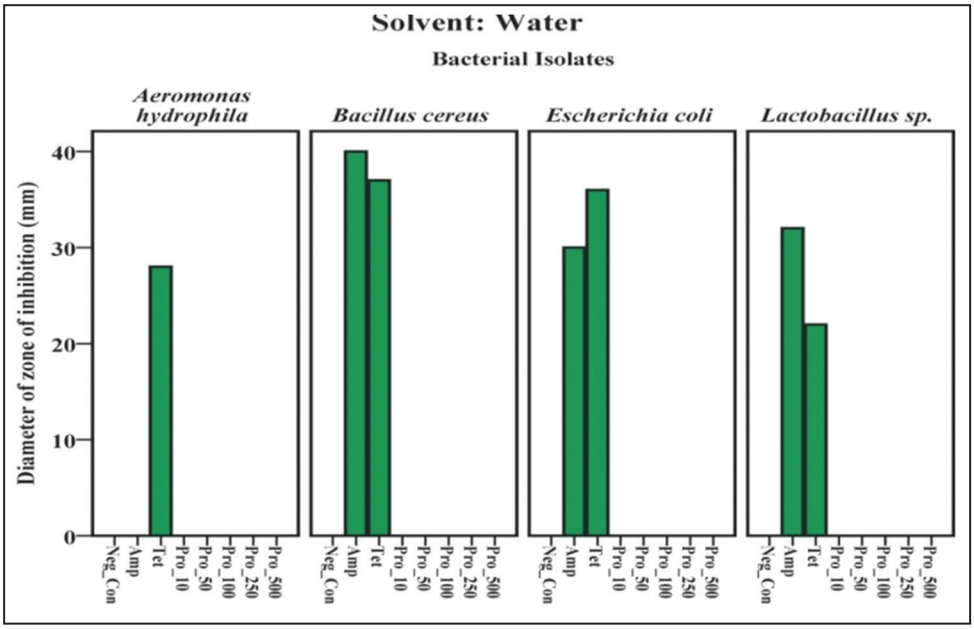


Figure 5: Antimicrobial activity of aqueous solution of probenecid Amp: Ampicillin (100 µg), Tet: Tetracyclin (50 µg), Pro_10: Probenecid (10 µg), Pro_50: Probenecid (50 µg), Pro_100: Probenecid (100 µg), Pro_250: Probenecid (250 µg), Pro_500: Probenecid (500 µg).

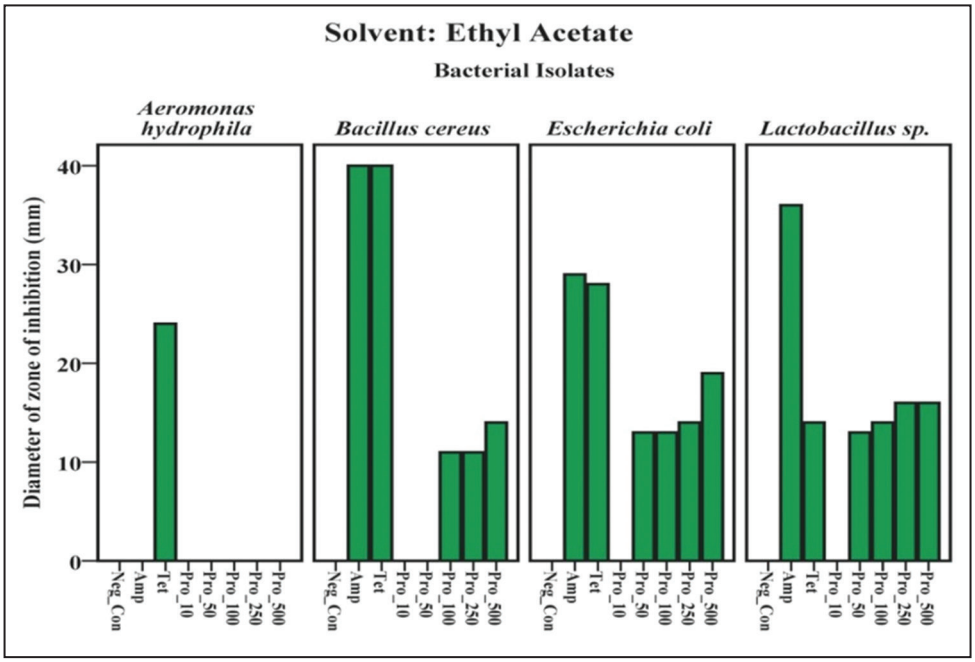


Figure 6: Antimicrobial activity of probenecid dissolved in ethyl acetate. Amp: Ampicillin (100 µg), Tet: Tetracyclin (50 µg), Pro_10: Probenecid (10 µg), Pro_50: Probenecid (50 µg), Pro_100: Probenecid (100 µg), Pro_250: Probenecid (250 µg), Pro_500: Probenecid (500 µg).

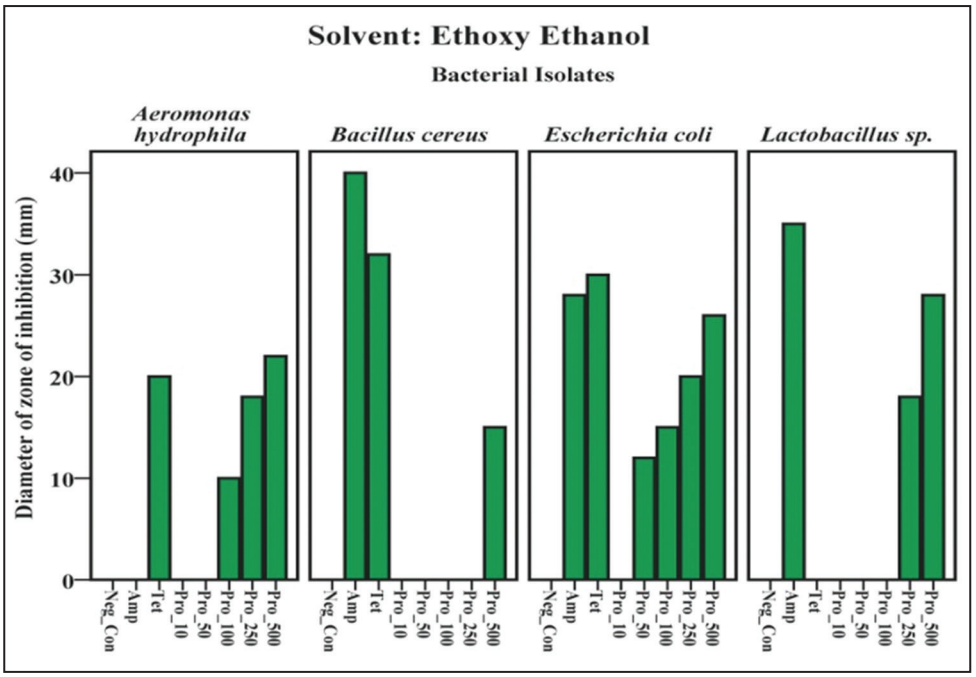


Figure 7: Antimicrobial activity of probenecid dissolved in ethoxyethanol. Amp: Ampicillin (100 µg), Tet: Tetracyclin (50 µg), Pro_10: Probenecid (10 µg), Pro_50: Probenecid (50 µg), Pro_100: Probenecid (100 µg), Pro_250: Probenecid (250 µg), Pro_500: Probenecid (500 µg).

3.5. Cell-viability and Cytotoxicity

In case of aqueous solution of probenecid no zone of inhibition was observed in all the four bacterial isolates. This may be ascribed to partial solubility of probenecid in water. Ampicillin and Tetracyclin (positive controls) showed the expected zones of inhibition (30 to 40 mm and 22 to 37 mm respectively) whereas water (negative control) showed no zone of inhibition at all. The result of this experiment is represented graphically in Figure 5. In case of probenecid dissolved in ethyl acetate zones of inhibition ranging from 13 to 19 mm were found whose diameters increased with increasing dose (Figure 6). Ampicillin and tetracycline showed zones of inhibition (29 to 40 mm and 14 to 40 mm respectively), whereas ethyl acetate (negative control) showed no zone of inhibition at all. The best zones of inhibition ranging from 12 to 28 mm were shown by probenecid dissolved in ethoxy ethanol whose diameter increased with increasing doses. Positive and negative controls showed zone of inhibition (20 to 40 mm) and no zone of inhibition respectively (Figure 7). Figure 8 represents a Mueller-Hinton agar plate showing growth of Escherichia coli (DH5α) being inhibited by probenecid dissolved in ethoxy ethanol, ampicillin and tetracyclin.

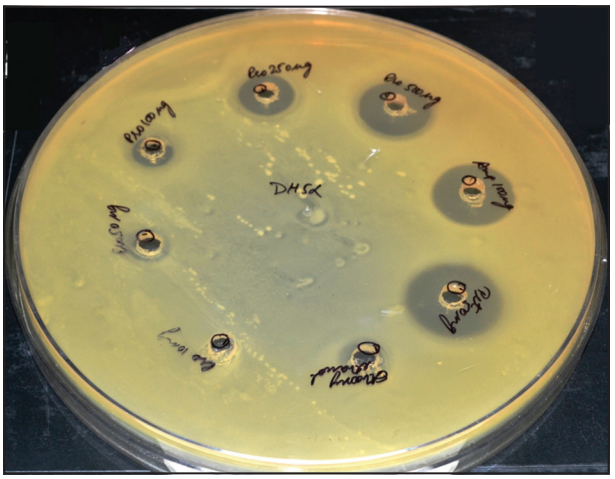


Figure 8: Mueller-Hinton agar plate showing growth of *Escherichia coli* (DH5α) being inhibited by probenecid dissolved in ethoxyethanol, ampicillin, and tetracycline.

4. RESULTS

In the case of the aqueous solution of probenecid, no zone of inhibition was observed in all the four bacterial isolates. This may be ascribed to partial solubility of probenecid in water. Ampicillin and tetracyclin (positive controls) showed the expected zones of inhibition (30–40 mm and 22–37 mm, respectively) whereas water (negative control) showed no zone of inhibition at all. The result of this experiment is represented graphically in Figure 5. In the case of probenecid dissolved in ethyl acetate, zones of inhibition ranging from 13 to 19 mm were found whose diameters increased with increasing dose (Figure 6). Ampicillin and tetracycline showed zones of inhibition (29–40 mm and 14–40 mm, respectively), whereas ethyl acetate (negative control) showed no zone of inhibition at all. The best zones of inhibition ranging from 12 to 28 mm were shown by probenecid dissolved in ethoxy ethanol whose diameter increased with increasing doses. Positive and negative controls showed a zone of inhibition (20–40 mm) and no zone of inhibition, respectively (Figure 7). Figure 4 represents a Mueller-Hinton agar plate showing growth of *E. coli* (DH5α) being inhibited by probenecid dissolved in ethoxy ethanol, ampicillin, and tetracycline.

5. DISCUSSION

Our results showed that probenecid dissolved in ethoxyethanol showed the maximum cytotoxic activity. This goes in unison with the result of the test of fluorescence which showed maximum fluorescence of this mixture among probenecid dissolved in water, ethyl acetate, and ethoxyethanol separately and also with its mixtures. Better

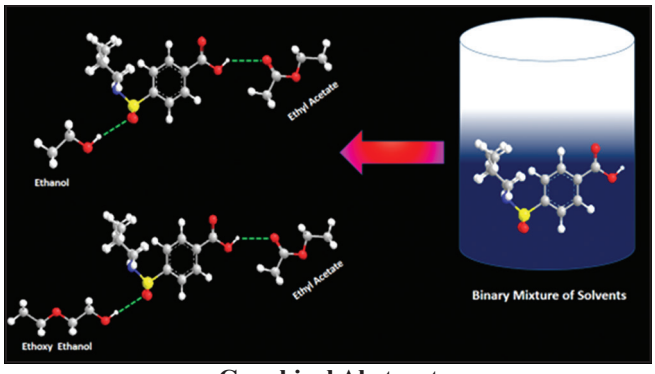
antimicrobial property of probenecid was observed against Gram-negative bacteria (*A. hydrophila* and *E. coli*) than against Gram-positive bacteria (*B. cereus* and *Lactobacillus*). Probenecid used along with other antibiotics has been shown to extend the plasma half life of beta-lactam antibiotics in the treatment of gonorrhea (Jensen *et al*., 1963). Our results prove that probenecid itself has some antimicrobial property [14-18].

6. CONCLUSION

This work authenticates that probenecid shows ion-solvent interaction with various types of solvents in both pure and mixed states. Probenecid which is a very important drug having various pharmaceutical applications used in this paper, to increase its novelty using various cosolvents. The interaction of PB with diverse solvents was studied by UV, steady-state fluorescence, conductivity, and IR measurements. These data depict that the interaction is more in the case of ethoxyethanol in both pure and mixed states. Furthermore, the drug showed the maximum cytotoxic activity with ethoxyethanol. PB is a water-insoluble drug that, when administered orally, is roughly completely absorbed into the bloodstream by means of the intestinal tract where it readily binds to plasma proteins, most prominently albumin. Hence, it can be inferred that ethoxyethanol and also their mixtures with ethyl acetate are better solvents in the miscibility and applicability of the drug.

7. ACKNOWLEDGMENT

Special Assistance in the paper is provided by "Rajiv Gandhi National Fellowship for Students with Disabilities 2015" and Department of Chemistry, NBU under the University Grants Commission, New Delhi for financial sustenance and instrumental facilities in order to carry out this research work.



Graphical Abstract

Highlights

1. Clinical uses and side effects of uricosuric drug is studied in this paper.
2. The slight preference of drug for ethoxyethanol in ethyl acetate-rich mixtures could be explained in terms of the common participation of basic sites in both solvents and/or the acidic site of ethoxy-ethanol with the respective counterparts of PB.
3. The experimental solubility results, cytotoxicity and models presented in the present work are essential in the practical process for production and purification, of PB.

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***Bibliographical Sketch**

Professor M. N. Roy is the senior most faculty of Chemistry, University of North Bengal, has research interest in the areas of Supramolecular Chemistry, Ionic Liquids, Solution Thermodynamics and Polymer Chemistry. He has travelled over many foreign countries for highlighting his research work. His contribution has been recognized by the publication of a large number of high impact 303 research articles including nature journals and 7 books. He is the life member of ICS, ISC, CRSI, and JTR. He has received prestigious "ONE-TIME GRANT", Rs.-7.00 lakh from UGC, New Delhi, under basic scientific research, "BRONZE MEDAL AWARD" from CRSI, "PROF. SURESH C AMETA AWARD" from ICS and "SHIKSHA RATNA AWARD" from the Government of West Bengal. Currently, he has been excellently and singly supervising eight PhD students and it is an immense pleasure to mention that thirty eight research scholars have already been awarded PhD degrees under his singly outstanding and proper supervision which is the highest record in the History of this University.