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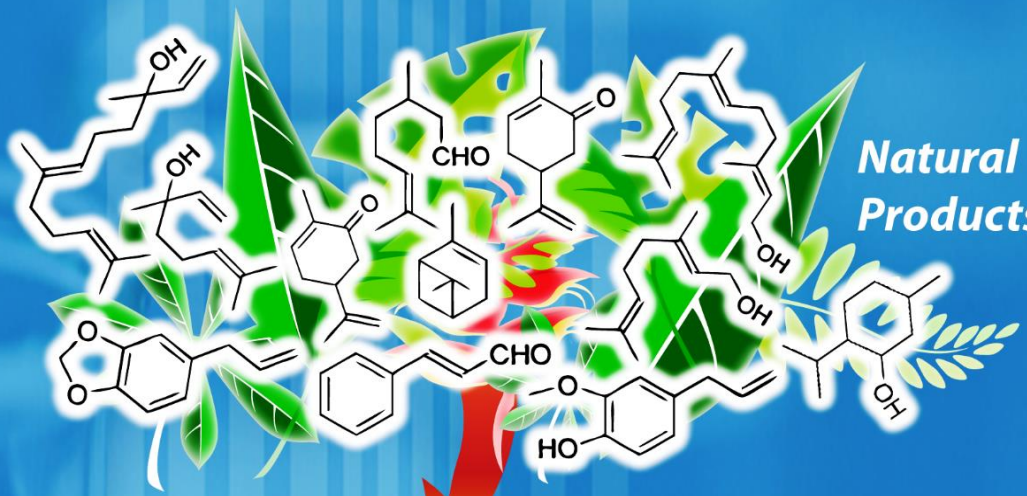

Aedes aegypti

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Editorial

Chemistry: A key science to fight neglected diseases

Over the past century, chemical and pharmaceutical industries have made substantial strides in the development of new pharmaceuticals for a range of human diseases. Discovering and developing new drugs are part of a costly process that involves not only the search for new drug candidates, but also the performance of clinical trials. It is not uncommon to find reports of costs reaching US$ 500 million—and even US$ 1 billion—engaging multidisciplinary teams for periods of 10 or 15 years. To a large extent, these costs are borne by consumers in richer countries. In contrast, diseases that predominantly affect residents of poorer nations tend to be left out of the modern process of drug discovery. Moreover, most of the world population stricken by these illnesses is incapable of supporting the costs involved in developing the drugs they need. Because these infirmities, with few exceptions, have been ignored by those in charge of research programs, both in the public and private sectors, they have been referred to as neglected diseases. On its website, the World Health Organization categorizes 14 health conditions as "tropical diseases," owing to their strict or predominant occurrence in the tropics. Most are infectious illnesses such as leishmaniasis, dengue fever, malaria, and Chagas' disease, which largely affect poor populations.

The drugs available for treating these conditions are regrettably scarce. In many regions, for instance, first-choice therapy for late-stage human African trypanosomiasis (HAT, or sleeping sickness) is still based on melarsoprol, an arsenic-derived drug developed in the late 19th century that can lead to fatal encephalopathy in 5% of patients. Another example is the unavailability of treatments, with the exception of the prolonged use of genotoxic drugs, for Chagas' disease, which affects a significant proportion of children.

Expansion of the arsenal of drugs for neglected diseases is therefore badly needed. The current situation calls for extensive studies and significant improvement in research support policies in the field of neglected diseases, despite the considerable body of scientific investigations focusing on the biology, immunology, and genetics of the parasites responsible for these conditions. Both in the university and the pharmaceutical industry, chemistry continues to drive the discovery of novel drugs. In the universities, chemists are contributing with studies targeted at obtaining compounds to fight neglected diseases. In this endeavor, the diverse areas of chemistry are increasingly teaming up to conduct studies so as to further clarify the structure–activity relationship of organic compounds; concurrently, increased knowledge is gained on the effects of small molecules on biological processes, thanks to the growing interaction between chemistry and biology. Today, chemists are no longer restricted to the realm of covalent bonds, but have also been exploring the territory of supramolecular interactions, constructing supermolecules that exhibit specific properties such as auto-assembly, molecular recognition, transformation, transport, and signaling [1]. In this new field, theoretical chemistry is crucial for the study of physicochemical properties, relying on quantum chemistry to anticipate the behavior or molecules and, consequently, their biological activity under different reactivity conditions [2]. Chemists still find their primary source of inspiration in natural products, as the wide range of structures exhibited by these compounds makes nature an inexhaustible source of new products—a range of diversity that

can astonish even the most resourceful chemist. No wonder, therefore, that nature's vast richness keeps continuously yielding useful products—in limitless numbers and for every conceivable purpose.

Química: Ciência central para combater doenças negligenciadas

Durante o século passado, indústrias químicas e farmacêuticas fizeram grandes progressos no desenvolvimento de novos fármacos para o tratamento de muitas doenças. O processo de descoberta e desenvolvimento de novos medicamentos, que engloba pesquisas para encontrar novos candidatos a fármacos e ensaios clínicos, é muito dispendioso. Não é raro encontrar referências sobre custos que variam de US$ 500 milhões a US$ 1 bilhão, envolvendo a dedicação de equipes multidisciplinares por períodos que alcançam de dez a quinze anos. Em grande medida, esses custos são bancados pelos consumidores de países mais ricos. Por outro lado, doenças que afetam predominantemente habitantes de nações mais pobres tendem a ser deixadas à margem do processo moderno de descoberta de drogas. Além disso, os atingidos são incapazes de arcar com os custos do desenvolvimento desses fármacos. Tais doenças têm sido ignoradas, com algumas exceções, pelos supervisores de programas de pesquisas do setor público ou privado e, por isso, são muitas vezes designadas como doenças negligenciadas. O site da Organização Mundial da Saúde lista 14 enfermidades na categoria "doenças tropicais", de ocorrência exclusiva ou majoritária nos trópicos. A maioria delas são doenças infecciosas que afetam principalmente populações pobres, como leishmaniose, dengue, malária e doença de Chagas.

Os medicamentos existentes para tratá-las são lamentavelmente poucos. Por exemplo, a terapia de primeira escolha destinada à fase 2 da tripanossomíase humana africana (HAT, ou doença do sono) em muitas regiões ainda é o melarsoprol (desenvolvido no final do século 19), que, por ser derivado de arsênio, provoca encefalopatia fatal em 5% das pessoas tratadas. Outro exemplo: o tratamento prolongado com drogas genotóxicas é a única escolha para a doença de Chagas, em que uma proporção significativa dos pacientes é constituída de crianças.

É urgente, portanto, a necessidade de ampliar o arsenal de medicamentos para as doenças negligenciadas. A situação exige amplas pesquisas e melhoria significativa da política de apoio à investigação na área das doenças negligenciadas, apesar do já considerável volume de trabalhos científicos relacionados à biologia, imunologia e genética dos parasitas que causam muitas dessas enfermidades. Tendo-se em vista a situação apresentada, o papel da química na academia e na indústria farmacêutica continua a ser o de propulsor decisivo para a descoberta de novas drogas. Na academia, os químicos contribuem com estudos voltados a obter compostos para combater doenças negligenciadas. Nesse campo, cada vez mais, as várias áreas da química se associam e desenvolvem estudos visando o entendimento da relação estrutura—atividade dos compostos orgânicos; simultaneamente a interação entre a química e a biologia permite compreender melhor os efeitos de pequenas moléculas em processos biológicos. Além disso, o químico de hoje está indo além das ligações covalentes, enveredando-se também no entendimento das interações supramoleculares, criando supermoléculas que apresentam propriedades específicas, tais como automontagem, reconhecimento molecular, transformação, transporte e sinalização [1]. Nesse contexto, a química teórica desempenha um papel de extrema

importância no estudo de propriedades físico-químicas, utilizando a química quântica para prever o comportamento de moléculas e, conseqüentemente, sua atividade biológica em diversas situações de reatividade [2]. Os produtos naturais são a maior fonte de inspiração para os químicos e abrangem uma variedade tão grande de estruturas, que permitem ver na natureza uma fonte inesgotável de novos produtos. Essa diversidade faz surpreender até mesmo os profissionais mais criativos da química. Não é de estranhar, portanto, que dentre toda essa riqueza seja possível encontrar produtos úteis para todos os fins, e em abundância.

Adilson Beatriz (UFMS)
Editor, Orbital

Dênis Pires de Lima (UFMS)
Associate Editor, Orbital

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[2] dos Santos, R.; Lacerda-Júnior, V. de Castro, E.; Balthar, V.; Greco, S. *Orbital* **2009**, *1*, 273. Available from: http://www.orbital.ufms.br/index.php/dqi/article/view/105/29

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Full Paper

A spectroscopic study of interaction of cationic dyes with heparin

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ABSTRACT: *The interaction of two cationic dyes namely, acridine orange and pinacyanol chloride with an anionic polyelectrolyte, heparin, has been investigated by spectrophotometric method.The polymer induced metachromasy in the dyes resulting in the shift of the absorption maxima of the dyes towards shorter wavelengths. The stability of the complexes formed between acridine orange and heparin was found to be lesser than that formed between pinacyanol chloride and heparin. This fact was further confirmed by reversal studies using alcohols, urea and surfactants. The interaction of acridine orange with heparin has also been investigated fluorimetrically.The interaction parameters revealed that binding between acridine orange and heparin arises due to electrostatic interaction while that between pinacyanol chloride and heparin is found to involve both electrostatic and hydrophobic forces. The effect of the structure of the dye in inducing metachromasy has also been discussed.*

Keywords: *cationic dyes, metachromatic complex, heparin, fluorescence quenching, structure, aggregation*

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Introduction

Metachromasy is a well-known phenomenon in the case of dye-polymer interactions and is generally found in the case of aggregation of cationic dyes on anionic polymers [1]. The metachromatic change, which is most pronounced in the visible spectrum, is frequently characterized by the appearance of a new absorption band. The change has been attributed due to the formation of various dye aggregates of the bound dye molecules on the polymer sites [2]. A similar change occurs with the cationic dyes in aqueous solution, as their concentration is increased and the temperature is decreased. The metachromatic changes are frequently specific rather than general and constitute an experimental basis of histo and cytochemical applications. For a particular polymer species it often depends on the specific substituent attached to the dyes even if they belong to a class of same chemical structure [3, 4]. The interaction of polyphospates and cationic dyes such as toludine blue [5], 4,5,4',5'-dibenzo-3,3'-diethyl-9-methyl-thiocarbocyanine [6], acridine orange and proflavine [7-11] are reported. The interaction of heparin with methylene blue and Azure A [12-16] are already reported in the literature. The phenomena of reversal of metachromasy by addition of urea, alcohols, neutral electrolytes [17] and also by increasing the temperature of the system, may be used to determine the stability of the metachromatic compound.

The fluorescence of the dye acridine orange (**1**) and its quenching in polymer matrices has been studied extensively [18-20]. Hence the objective of the present study is to understand the forces involved in the binding of the dyes to the anionic polyelectrolyte and also to evaluate the thermodynamic parameters of interaction. Moreover, pinacyanol chloride (**2**) is larger in size and would yield better information on dye polymer interaction. Hence it has been selected for our present studies. The equivalent weight of heparin was found to be 178 [21-24].

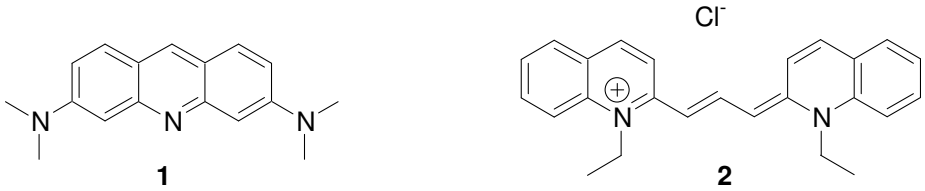

Figure 1. Structures of acridine orange (**1**) and pinacyanol chloride (**2**).

Material and Methods

Reagents

Acridine orange (AO), was obtained from S. D. Fine Chemicals, Mumbai and used as received. Pinacyanol chloride (Pcyn) was obtained from Acros media and used as received. Sodium heparinate (NaHep) (Lobachemie, India) was used as received; Methanol, ethanol and 2-Propanol (Merck, India) were distilled before use. Urea, sodium chloride, potassium chloride, sodium lauryl sulphate and sodium dodecyl benzene sulphonate (Loba Chemie, India) were used as received. Absorption Spectra were recorded using a Shimadzu UV-2550 Spectrophotometer. The concentrations of the stock solution of the dye and the polymer were $6x10^{-5}$ M and $1x10^{-3}$ M, respectively.

Determination of stoichiometry of polymer-dye complex

Increasing amounts of polymer solution (0.0-9ml,$1x10^{-3}$ M) were added to a fixed volume of dye solution(0.6mL, $1x10^{-3}$ M) in case of acridine orange and (0.5mL,$1x10^{-3}$ M) in different sets of experiments and the total volume was made up to 10 mL by adding distilled water in each case. The absorbances were measured at the respective monomeric and metachromatic bands.

Effect of alcohols and urea on the absorption of pure dye

The effect of alcohols namely, methanol, ethanol, 2-propanol and urea on the spectra of the pure dye was studied by measuring absorbance of the pure dye solution at 492 nm in case of acridine orange and at 600 nm in case of pinacyanol chloride in the presence of alcohols and urea. 10 mL solutions were made with 0.6 mL of $1x10^{-3}$ M dye and remaining alcohol (10-80%) or aqueous urea (1-8 M) and the absorbances were recorded at the respective monomeric band.

Study of reversal of metachromasy using polymer-dye complex

For measurements of the reversal of metachromasy, solutions containing polymer and dye in the ratio 2.0:1.0 were made containing different amount of alcohol. The total volume was maintained at 10 mL in each case. The absorbances were measured at 492 nm and 457 nm in case of acridine orange and at 600 nm and 486 nm in case of pinacyanol chloride. Similarly, polymer-dye solutions containing different amounts of urea (1-8 M) were made and the absorbances were measured at 492 nm and 457 nm in case of acridine orange–Heparin complex and at 600 nm and at 486 nm in case of Pinacyanol chloride-Heparin complex.

Fluorescent studies

Fluorescence of the dye solutions as well as that of the dye-polymer mixture was measured using Jasco FP-6200 Spectrofluorometer. Spectrofluorometric titrations were carried out by measuring fluorescence intensity of the AO solutions, at the emission peak, 529 nm upon addition of increasing amount of polymer sample under separate experiments. The concentration of the aqueous solution of the dye was $6x10^{-5}$ M. The

concentration of the polymer solution varied from 10^{-3} M-10^{-4} M. Increasing amounts of polymer solution were (0.0mL-9 mL, 1x10^{-3} M) added to fixed volume of dye solution in different sets of experiments and the total volume was made upto 10 mL in different sets of experiments and the total volume was made up to 10 mL by adding distilled water in each case.

Determination of thermodynamic parameters

The thermodynamic parameters were determined by measuring the absorbances of the pure dye solution at both the respective monomeric and metachromatic band in the temperature range (36-54 ^{0}C).The above experiments ware repeated in presence of the polymer at various polymer-dye ratios (2, 5, 10, and 20).

Determination of equivalent weight of heparin

The given sample of sodium heparinate was completely converted to heparinic acid. To ensure complete conversion of sodium heparinate to heparinic acid, each sample was passed through a column of approximately 50-fold excess Dowex-50W (H^+, form, x 4, 200 – 400 mesh) immediately before titration. The possibility of hold up of heparin on the column was eliminated by subsequent passage of ten-fold excess, over heparin, of 4 N [21-23]. The ratio of number of sulphate to carboxylate groups in heparin was determined by conductometric titration method [24]. The equivalent weight of heparin was found to be 178.

Results and Discussion

The absorption spectra of acridine orange (**1**) and pinacyanol chloride (**2**) at various concentrations are shown in figures 2 and 3, respectively. The maxima absorption was found to be 492 nm in case of acridine orange and 600 nm in case of pinacyanol chloride, indicating the presence of a monomeric dye species in the concentration range studied. On adding increasing amounts of polymer solution the absorption maxima shifts to 457 nm in case of AO-Heparin and at 486 nm in case of pincyanol chloride-heparin complex. The blue shifted band is attributed to the stacking of the dye molecules on the polymer backbone and this reflects high degree of co-operativity in binding [25]. Appearance of multiple banded spectra proposed that the polymer might have a random coil structure in solution. Whereas at higher concentration of the polymer almost a single banded spectrum was observed due to possible change from random coil to helical form. In this regard the accepted mechanism suggests that the dye cations bind to adjacent sites of the polymer forming a single individual compound, which is considered to arise from electrostatic interaction among neighboring

dye molecules and fixed sites on polymer as a result of which they suffer effective aggregation resulting in hypochromic and hypsochromic spectral shifts in the absorption band of the dye [26]. The results are shown in figures 4 and 5, respectively.

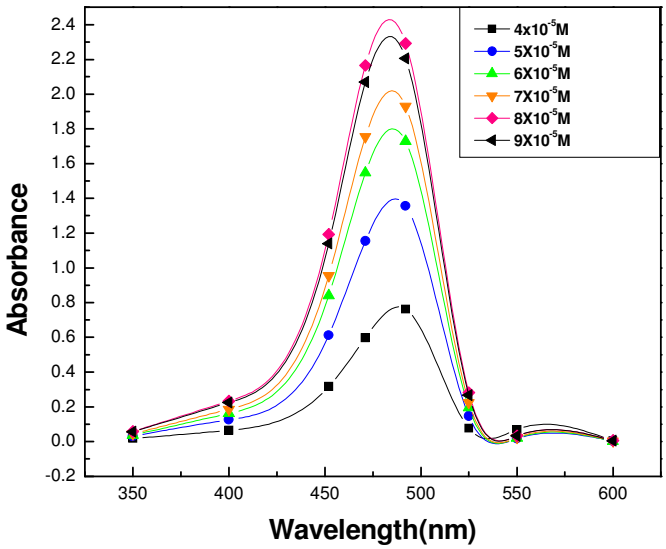

Figure 2. Absorption spectrum of dye, acridine orange at various concentrations.

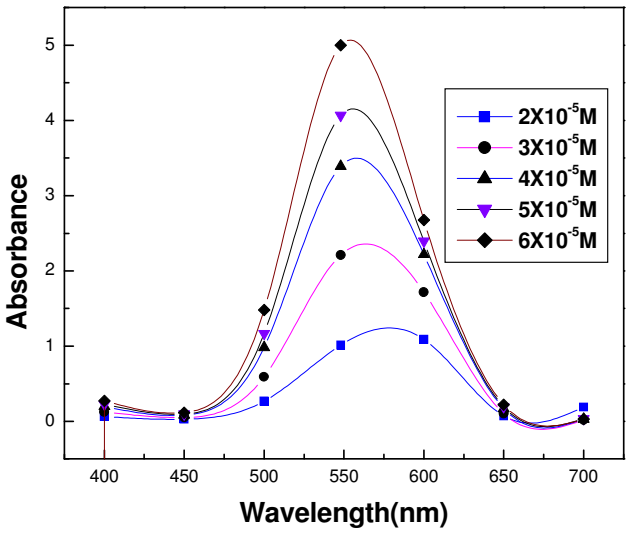

Figure 3. Absorption spectrum of dye, pinacyanol chloride at various concentrations.

Determination of stoichiometry

To determine the stoichiometry of the polymer-dye complex, a plot of A_{457}/A_{492} versus the polymer/dye ratio was made. The stoichiometry of AO-NaHep complex was found 1:1.5 which indicates that the binding is at adjacent anionic sites. This

stoichiometry indicates that every potential anionic site of the polyanion was associated with the dye cation and aggregation of such dye molecules was expected to lead to the formation of a card pack stacking of the individual monomers on the surface of the polyanion so that the allowed transition produces a blue-shifted metachromasy [27]. While in case of pinacyanol chloride-heparin complex the stoichiometry is 1:2 and the binding is at alternate anionic sites. This indicates that there is lesser overcrowding and more aggregation of the bound dyes on the polymer chain in the latter case than in the former case. Similar results were reported in case of binding of pinacyanol chloride on poly(methacrylic acid) and poly(styrene sulfonate) systems [28].The results are shown in figures 6 and 7, respectively.

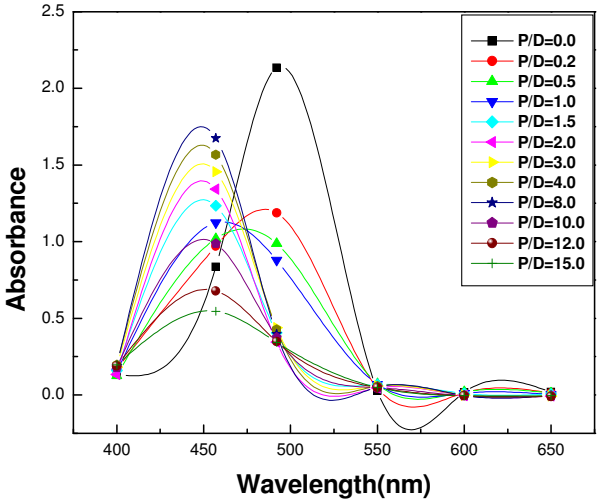

Figure 4. Absorption spectrum of AO-NaHep system at various P/D ratios.

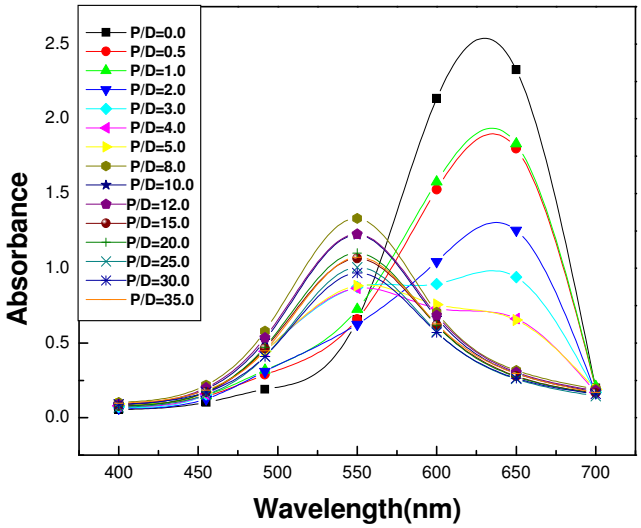

Figure 5. Absorption spectrum of pinacyanol chloride-NaHep system at various P/D ratios.

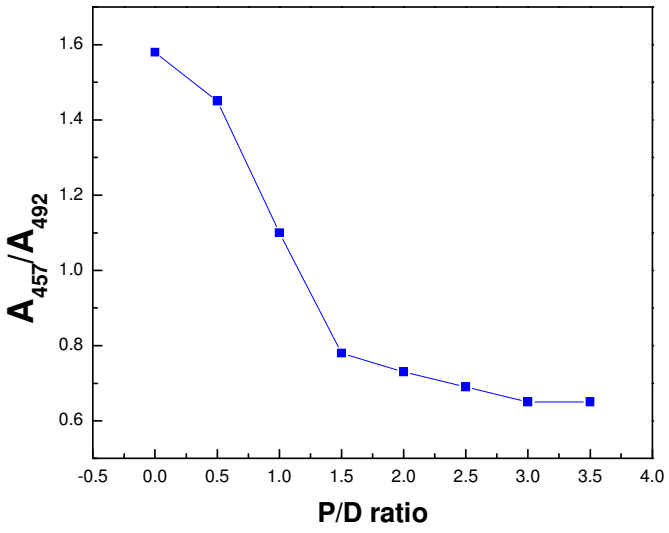

Figure 6. Stoichiometry of AO-NaHep complex.

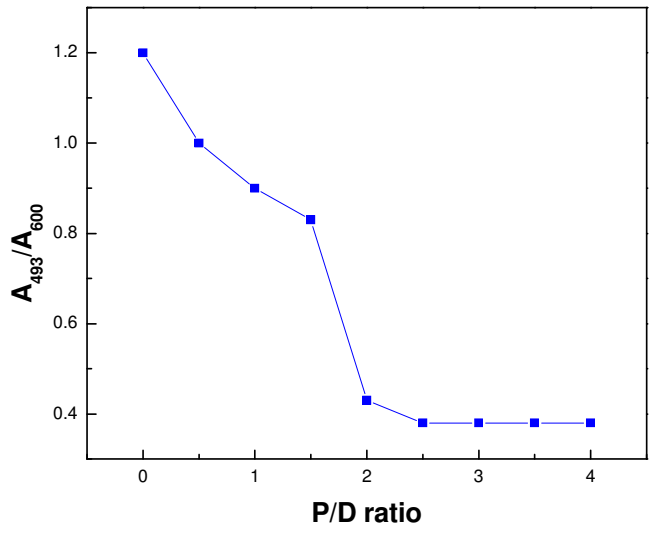

Figure 7. Stoichiometry of Pcyn-NaHep complex.

Reversal of metachromasy using alcohols and urea*:*

The metachromatic effect is presumably due to the association of the dye molecules on binding with the polyanion which may involve both electrostatic and hydrophobic interactions. The destruction of metachromatic effect may occur on addition of low molecular weight electrolytes, alcohols or urea. The destruction of metachromasy by alcohol and urea is attributed to the involvement of hydrophobic bonding has already been established [29-32]. The efficiency of alcohols in disrupting metachromasy was

found to be in the order methanol<ethanol<2-propanol, indicating that reversal becomes quicker with increasing hydrophobic character of the alcohols. The above facts are further confirmed established in the present system. On addition of increasing amount of alcohol to the polymer/dye system at P/D=2.0, the original monomeric band of dye species is gradually restored. The efficiency of the alcohols, namely methanol, ethanol and propanol, on destruction of metachromasy was studied as shown in figures 8 and 9, 50% methanol, 40% ethanol, 30% 2-propanol were required to reverse metachromasy in P-heparin system. While in case of AO-Heparin system 50% methanol, 25% ethanol, 20% 2-propanol were sufficient to reverse metachromasy. However, the concentration of urea to reverse metachromasy is found to be as high as 5 M in P-Hep system and 4 M in case of AO-Hep system (figures 10 and 11). Similar reports are available in literature for reversal of metachromasy in anionic polyelectrolyte/cationic systems by addition of alcohols or urea [33-37].

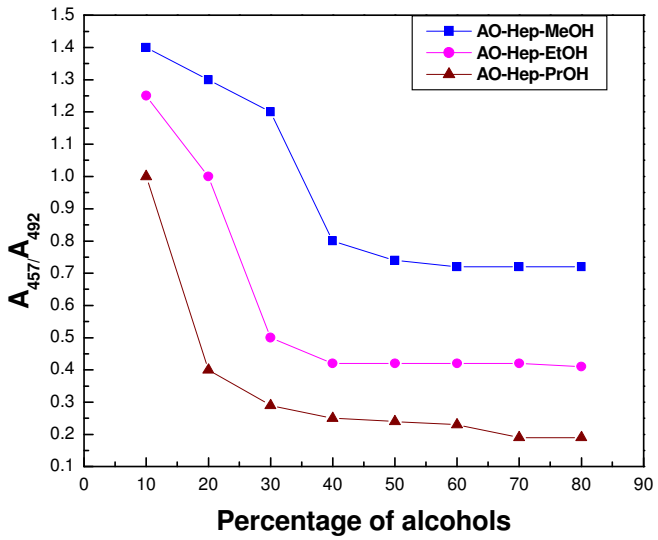

Figure 8. Reversal of metachromasy on addition of alcohols in AO-Hep systems.

Effect of surfactants

The strength and nature of interaction between water soluble polyelectrolyte and oppositely charged surfactants depend on the characteristic features of both the polyelectrolyte and the surfactant. The charge density, flexibility of the polyelectrolyte and the hydrophobicity of the non-polar part and the bulkiness of the polar part also play a vital role in the case of polysaccharide-surfactant interaction [38]. On adding increasing amounts of sodium laurylsulphate and sodium dodecylbenzene sulphonate to AO-sodium heparin complex the molar concentrations of sodium laurylsulphate and sodium dodecylbenzene sulphonate needed to cause reversal was found to be $1x10^{-3}$ M and $1x10^{-}$

[4] Min case of AO-Hep and $1x10^{-3}$ M and $1x10^{-2}$ M in case of P-Hep system. These results agree with those reported earlier in literature [39] .Thus the addition of surfactants causes the production of micelles. Thus the surfactant molecules interacted with the polymer by replacing the cationic dye. The release of dye molecules from the dye-polymer complex in presence of cationic surfactants revealed that surfactants interacted electrostatically [40] with the anionic site of the polymer and thus the dye becomes free. The ease of reversal of metachromasy can be correlated with its chain length [41]. Thus the binding between oppositely charged polymer surfactant is primarily electrostatic force which is reinforced by hydrophobic forces. The results are shown in figures 12 and 13, respectively.

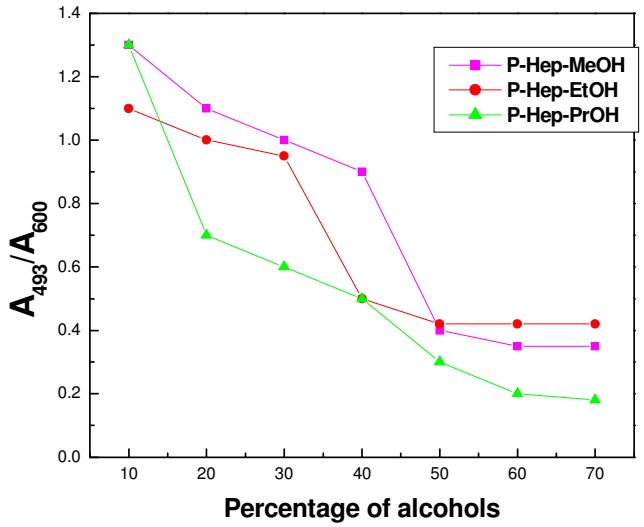

Figure 9. Reversal of metachromasy on addition of alcohols in Pcyn-Hep systems.

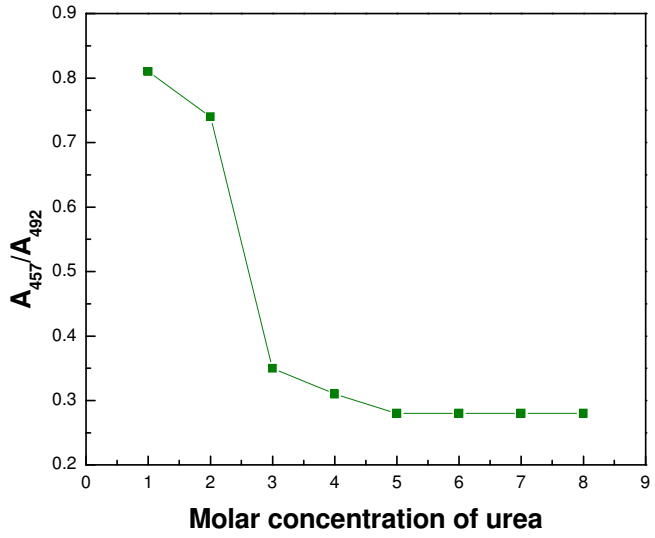

Figure 10. Reversal of metachromasy on addition of urea in AO-Hep complex.

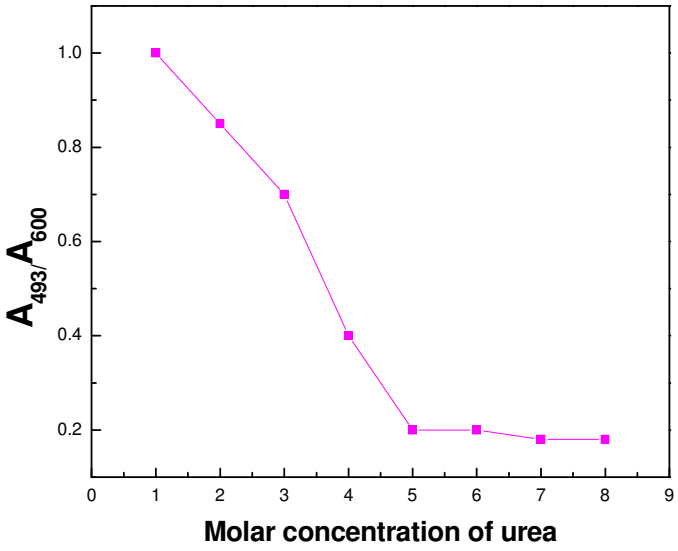

Figure 11. Reversal of metachromasy on addition of urea in Pcyn-Hep complex.

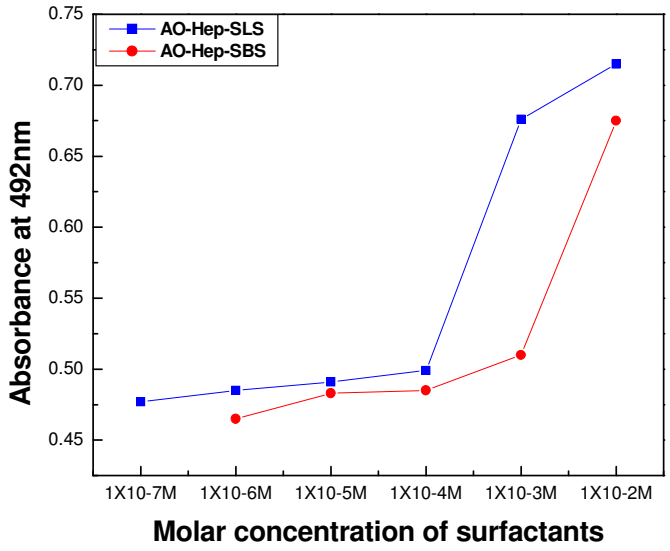

Figure 12. Reversal of metachromasy on addition of surfactants in AO-Hep complex.

Effect of ionic strength:

Tan & Schneider [42] have reported the disruption of metachromatic band with the variation of ionic strength. When sodium chloride of varied ionic strength were added to AO-Hep and Pcyn-Hep complex and the absorbances were measured in the range 350-700 nm. In case of Pcyn-Hep the monomeric band reappears at higher ionic strength (0.1 M) than compared to that of AO-Hep ($1x10^{-3}$ M). In aqueous solutions the charged polymer molecule will be in the extended conformation due to the repulsion between the charged groups. On adding the dye the conformation of the polycation, changes to a

compact coil owing to dye binding, resulting from electrostatic interaction thus giving rise to metachromatic band. The concentration of sodium chloride required to reverse metachromasy was greater in case of pincyanolcloride-heparin complex than in the case of acridine orange-heparin complex. The results are shown in figures 14 and 15, respectively.

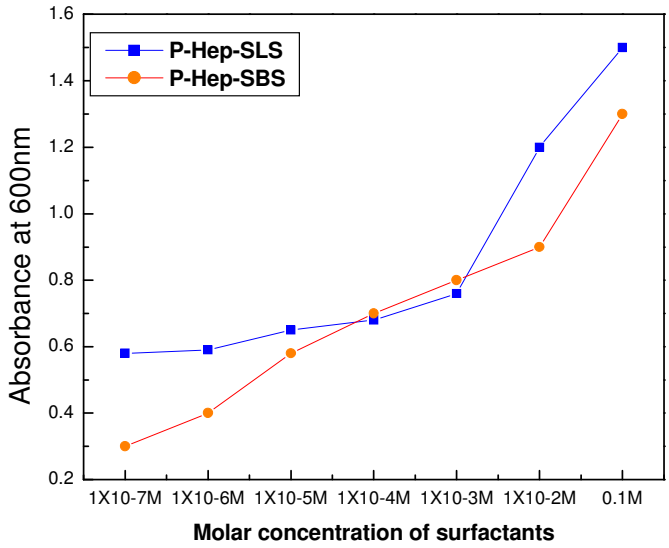

Figure 13. Reversal of metachromasy on addition of surfactants in Pcyn-Hep complex.

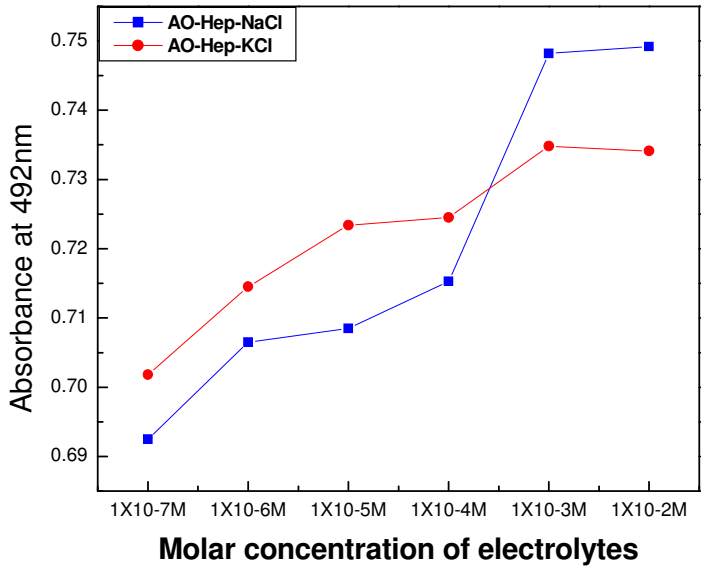

Figure 14. Reversal of metachromasy on addition of electrolytes in AO-NaHep complex.

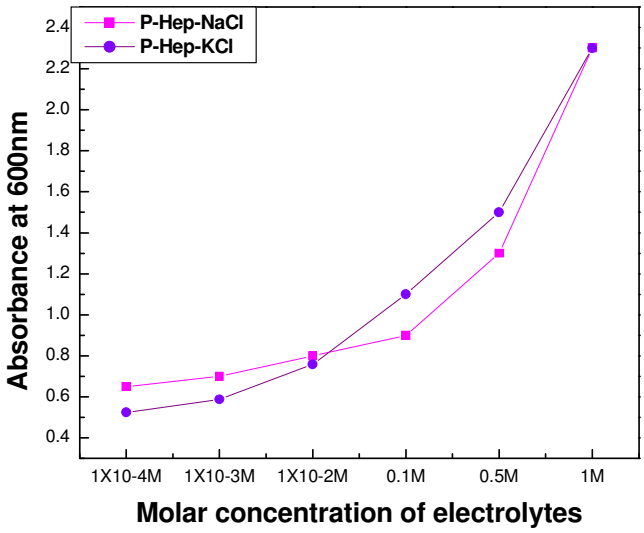

Figure 15. Reversal of metachromasy on addition of electrolytes in Pcyn-NaHep complex.

Determination of interaction parameters

The interaction constant K_c for the complex formation between AO and NaHep and Pcyn-NaHep was determined by absorbance measurements at the metachromatic bands at four different temperatures taking different sets of solutions containing varying amounts of polymer (C_S) in a fixed volume of the dye solution. The value of K_c was obtained from the slope and intercept of the plot of $C_DC_S/ (A-A_O)$ against Cs shown in figures 16 and 17. Absorbance results were treated using Rose-Drago eqn. [43], $\mathbf{C_D.Cs/(A-A_0) = 1/K_cL\ (\varepsilon_{ds}- \varepsilon_d) + Cs/L\ (\varepsilon_{ds}- \varepsilon_d)}$. $\mathbf{C_D}$ refers to the initial molar concentration of dye and $\mathbf{C_S}$ refers to the concentration of the polymer sample. In each case the thermodynamic parameters of interaction, namely ΔH, ΔG and ΔS were also calculated (figures 18 and 19). The results are given in the Table 1.

a) Calculated from figures 16 and 17 according to Rose-Drago equation.

b) Calculated from the thermodynamic equation $\Delta G = -RT\ln K_c$.

c) Calculated graphically by plotting $\ln K_c$ against 1/T according to Van´t Hoff equation,

$\ln K_c = -\Delta H/RT + C$.

d) Calculated from the thermodynamic expression $\Delta G = \Delta H - T\Delta S$.

The values of interaction constant of AO-NaHep and Pcyn-NaHep decreases with rise in temperature suggesting the exothermic nature of the reactions and such a low

value ΔG suggested a non-chemical type of interaction. The negative value of entropy indicated a more ordered state of the ions due to its aggregation. Thus all these thermodynamic parameters suggested the interaction between the anionic sites of the polyanions and the counter ions resulting in aggregation and induction of metachromasy.

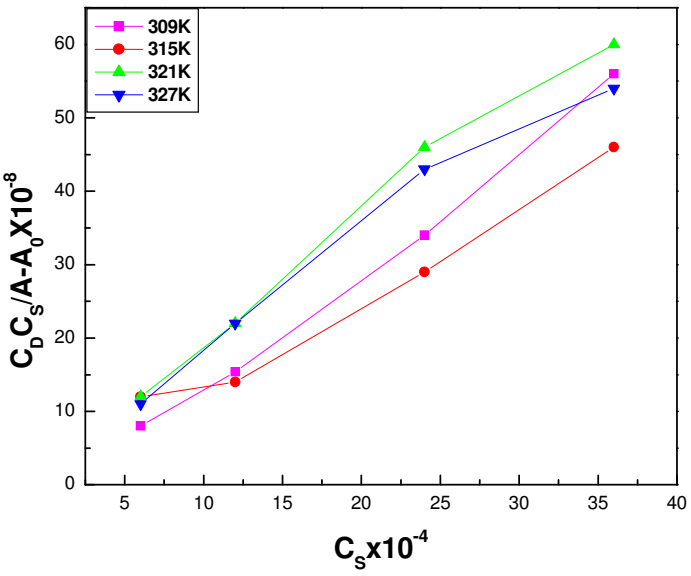

Figure 16. Plots of $C_D.C_S/(A\text{-}A_O)$ against Cs for AO-NaHep system at different temperatures.

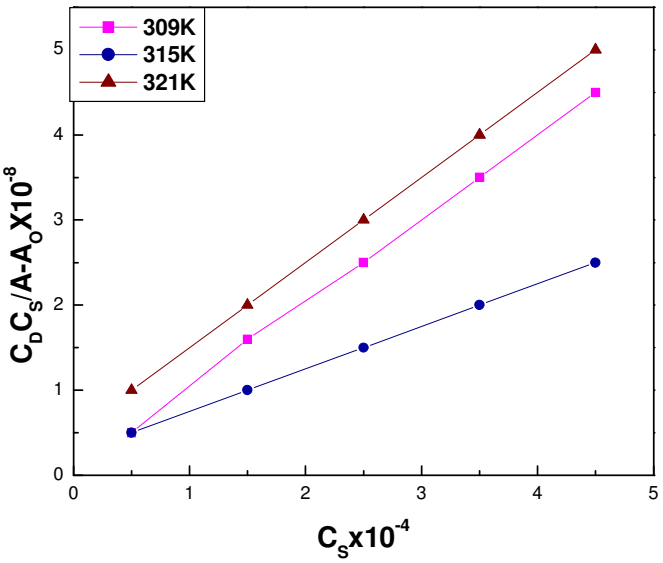

Figure 17. Plots of $C_D.C_S/(A\text{-}A_O)$ against Cs for Pcyn-NaHep system at different temperatures.

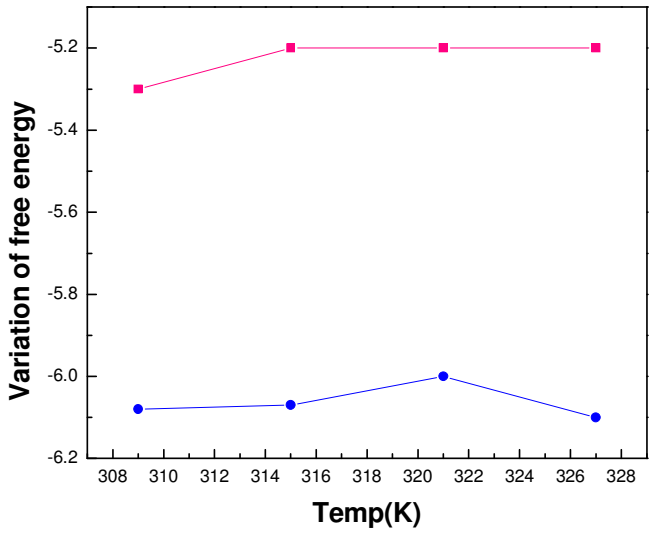

Figure 18. Variation of ΔG with temperature in AO-NaHep and Pcyn-NaHep systems.

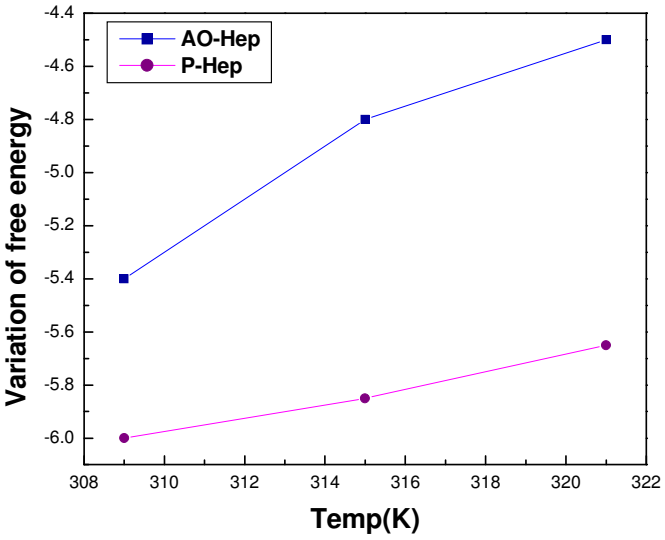

Figure 19. Van't Hoff plots for AO-NaHep and Pcyn-NaHep systems.

Fluorescence measurements

Fluorescent studies were performed with AO-NaHep system and it was found that the fluorescent intensity of AO decreased on the addition of increasing amounts [43] of polymer solution as evidenced from Figure 20. This indicated that the planar cationic dye AO preferred to form aggregates of dye molecules on binding to heparin. Such fluorescence quenching of AO by various bacterial polysaccharides are already reported

in the literature [44]. Finally, to study the interaction between the polymer and dye, the fluorescence data were fitted to Stern-Volmer equation, Fo/F=1+Ksv[Q] , where Fo is the fluorescence intensity of the dye solution and F is that of the dye-polymer mixture, and [Q] indicated the molar concentration of the polymer, Ksv is the Stern-Volmer constant. The Stern-Volmer plots obtained for the present data is shown in Figure 21. From the slope of the plot of F_O/F the value of the Stern-Volmer plot found to be $1.1x10^3$ $lit^{-1}mol^{-1}$, which is less than that reported for the interaction between AO-Klesiella. One possible explanation for that may be due to the self-association of quencher molecule, or the quencher with the fluorescer as reported in the literature. The linearity of the plot indicated that the quenching in this case is static in nature [45].

Table 1. Thermodynamic Parameters for interaction of AO-NaHep and Pcyn-NaHep

Temp	**KC(dm^3 mol^{-1})[a]**		**ΔG(kcal.mol^{-1})[b]**		**ΔH (kcal. mol^{-1})[c]**		**ΔScal.mol^1K^{-1})[d]**	
(K)	**AO-Hep**	**P-Hep**	**AO-Hep**	**P-Hep**	**AO-Hep**	**P-Hep**	**AO-Hep**	**P-Hep**
309	5400	20000	-5.3	-6.08				
315	3500	16666	-5.29	-6.07	-6.724	-7.316	- .0046	-.004
321	1600	13820	-5.25	-6.0				
327	1062	10020	-5.22	-6.1				

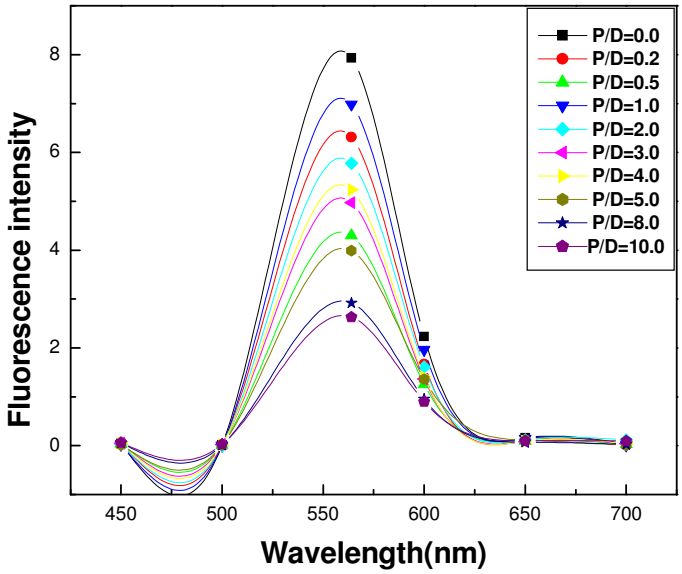

Figure 20. Emission spectra of AO –NaHep system at various P/D ratios.

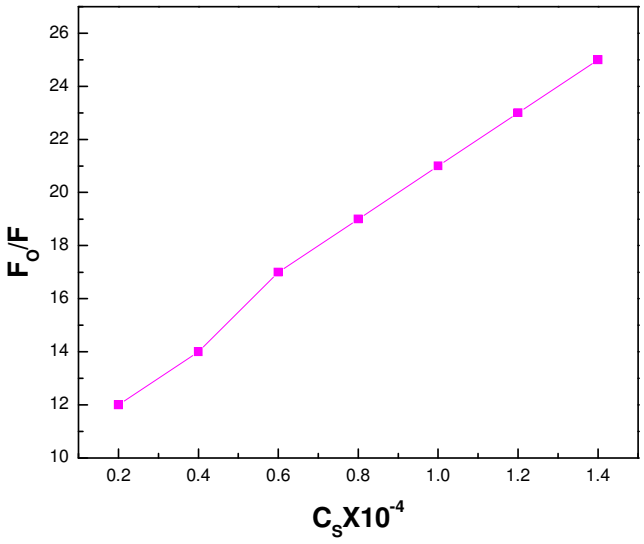

Figure 21. Stern-Volmer plot for AO-NaHep complex.

Effect structure of the dye

The structures of acridine orange and pinacyanol chloride are given at Figure 1. Thus it is evident that acridine orange, being a rigid planar cationic dye and hence a small distance between the adjacent anionic sites on the polyanion will be more favorable for binding resulting in stacking arrangement. On the other hand, pinacyanol chloride being larger in size is more hydrophobic and hence induces greater aggregation. Thus in this case the distance between the two adjacent dye molecules will be greater and the dye molecules are oriented like a stair case which agrees with the reported literature [46]. Furthermore the dye molecules are arranged in a parallel and stacked manner within the aggregates resulting in a hypsochromic shift [47].

Conclusion

The polymer heparin is observed to be effective in inducing metachromasy in the dyes AO and pinacyanol chloride. The metachromatic spectral shift is 35 nm in case of AO-Heparin and 114 nm in case of P-Heparin this can be correlated to the structure of the dye. Thus pinacyanol chloride, a cyanine dye is more hydrophobic and more aggregating in nature. The 2:1 stoichiometry indicates binding is at alternate anionic sites in case of P-Hep whereas in case of AO-Heparin the binding is at adjacent anionic sites. The study of the effect of alcohols and urea on the reversal of metachromasy indicates weak electrostatic binding between AO-NaHep, which is evident from the

thermodynamic data and fluorescence study. Thus the binding between Pcyn-NaHep is governed by strong electrostatic interaction reinforced by hydrophobic interaction. Thus the spectral studies prove the chromotropic character of NaHep and the cooperative nature of the dye-polymer interactions in various systems.

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Full Paper

Uso de métodos quimiométricos e mecânico-quanticos na análise de terpenóides e fenilpropanóides bioativos contra o *Aedes aegypti*

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RESUMO: *O dengue é um dos principais problemas de saúde pública no mundo. Muitos mosquitos já apresentam resistência aos inseticidas convencionais utilizados. Assim, tem-se aumentada a procura por extratos vegetais e substâncias naturais como inseticidas alternativos. Neste trabalho, métodos quimiométricos foram empregados para classificar um conjunto de compostos terpenóides e fenilpropanóides com atividade biológica contra as larvas do mosquito A. aegypti. O método AM1 (Austin Model 1) foi utilizado para calcular um conjunto de descritores moleculares (propriedades) para os compostos em estudo. A seguir, os descritores foram analisados utilizando os seguintes métodos de reconhecimento de padrões: Análise de Componentes Principais (PCA) e Análise Hierárquica de Agrupamentos (HCA). Os métodos PCA e HCA mostraram-se bastante eficientes para classificação dos compostos estudados em dois grupos (ativos e inativos). As variáveis eletrônicas* E_{HOMO-1}, E_{HOMO-2}, E_{LUMO}, E_{LUMO+2} *e a estrutural LogP foram responsáveis pela separação entre os compostos ativos e inativos. Na maioria dos compostos estudados, as variáveis responsáveis pela separação entre compostos ativos e inativos foram descritores eletrônicos, podendo-se concluir que efeitos eletrônicos desempenham papel fundamental na interação entre receptor biológico e compostos terpenóides e fenilpropanóides com atividade contra as larvas do mosquito A. aegypti.*

Palavras-chave: *terpenos, dengue, AM1, quimiometria, PCA*

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Introdução

O dengue é uma doença reemergente que vem preocupando as autoridades sanitárias de todo o mundo em virtude de sua circulação nos cincos continentes e pelo grande potencial para assumir formas graves e letais. O vírus do dengue é um arbovírus do gênero *Flavivírus*; é transmitido por mosquitos do gênero *Aedes*, sendo o *Aedes aegypti* seu principal vetor. São conhecidos quatro sorotipos do vírus [1- 4].

Como ainda não existem vacinas disponíveis contra os diferentes sorotipos da doença, o principal meio de controle do dengue baseia-se no combate ao *Aedes aegypti*, por meio do saneamento do meio ambiente, eliminação dos focos de procriação do vetor e proteção individual contra picadas. As ações de combate podem ser focadas nos mosquitos imaturos (formas aquáticas), por meio do controle físico, controle químico e controle biológico, ou nos mosquitos adultos, por meio de inseticidas, repelentes e barreiras mecânicas, ou ainda em ambos [3, 5].

O controle do vetor utilizando inseticidas sintéticos constitui a principal medida adotada pelos programas de saúde pública [4]. O uso contínuo desses inseticidas tem se mostrado eficaz no processo de erradicação, mas não obstante tem conduzido cada vez mais ao desenvolvimento de larvas e adultos resistentes; o odor do produto é desagradável e o princípio ativo costuma ser danoso às pessoas que sofrem de problemas alérgicos e respiratórios [5, 6].

A resistência aos inseticidas convencionais surge hoje como um dos principais obstáculos ao controle de insetos de importância na agricultura e na medicina. Dados da Organização Mundial de Saúde revelam que o custo da resistência de insetos a inseticidas pode alcançar anualmente 1,4 bilhões de dólares nos Estados Unidos [7].

A procura por metodologias naturais e menos agressivas aos seres humanos tem crescido consideravelmente nos últimos anos. Uma alternativa ao controle químico convencional é a utilização de extratos vegetais e substâncias naturais efetivas no controle do mosquito adulto e/ou da larva do *Aedes aegypti* e que sejam isentas de toxicidade para o meio ambiente [8].

Muitos dos metabólitos secundários produzidos pelas plantas são usados pelas mesmas contra microrganismos e insetos predadores, o que as torna potenciais candidatas para a descoberta de novos produtos contra o *Aedes aegypti*. Como exemplo tem-se a ação repelente dos óleos essenciais de casca de laranja [9], tomilho e cravo [10] e substâncias como eugenol, cineol e citronelal [11]. Estudos com *Lippia sidoides* (alecrim pimenta) [12] e *Cymbopogon citratus* (capim limão) [4] sugerem que seus óleos essenciais possuem ação larvicida contra o *Aedes aegypti*.

Plantas, como organismos que co-evoluem com insetos e outros microrganismos, são fontes naturais de substâncias inseticidas e antimicrobianas, já que as mesmas são produzidas pelo vegetal em resposta a um ataque patogênico [13]. As plantas sintetizam e liberam inúmeras substâncias voláteis para atrair polinizadores e se defender de patógenos. Terpenos e fenilpropanóides, principais constituintes dos óleos essenciais [14], sintetizados por espécies vegetais podem ter, dependendo do inseto em análise, propriedades atrativas (alimentação, polinização) e/ou repelentes e inseticidas.

Nos últimos anos, óleos essenciais obtidos de plantas têm sido considerados fontes em potencial de substâncias biologicamente ativas [15]. Ênfase tem sido dada às propriedades antimicrobiana, antitumoral, larvicida, repelência e inseticida de compostos voláteis.

Com relação ao controle do mosquito vetor do dengue, os esforços têm se concentrado no combate às larvas do *Aedes aegypti*. Trabalhos relatados na literatura mostraram que alguns óleos essenciais apresentaram expressivas atividades larvicidas contra espécies de mosquitos como *Culex quinquefasciatus* [16, 17] e *Aedes aegypti* [4, 12, 18, 19].

Os monoterpenos e os fenilpropanóides, compostos encontrados abundantemente nos óleos essenciais e, tipicamente lipofílicos, apresentam alto potencial para interferências tóxicas em processos bioquímicos básicos, com consequências, fisiológicas em larvas e comportamentais em insetos [14, 20].

Os óleos essenciais são larvicidas e inseticidas de ação rápida, indicativa de modo de ação neurotóxica, com evidências de interferência no neuromodulador octopamina ou em canais de cálcio. A octopamina é semelhante à noradrenalina, agindo como um neurormônio, neuromodulador e neurotransmissor [21]. Podem, também, apresentar ação larvicida e inseticida em função da inibição da enzima acetilcolinesterase [22] além da inibição do citocromo P450 monoxigenase-dependente e ação no sistema nervoso octopaminérgico [23].

Todos esses mecanismos de ação ainda não são completamente compreendidos e determinados sendo que, estudos que proporcionam uma melhor elucidação da relação estrutura-atividade se fazem necessário. O estudo de propriedades físico-químicas utilizando a química quântica é importante para prever o comportamento químico de moléculas e, consequentemente, a sua atividade biológica em diversas situações de reatividade.

Os métodos quânticos semi-empíricos são baseados no mesmo formalismo dos métodos *ab initio*, mas parte de seus parâmetros são ajustados a dados

experimentais. A parametrização dos métodos semi-empíricos com dados experimentais aumentou significativamente a acuracidade química e a velocidade dos métodos de orbitais moleculares. O sucesso desta abordagem é indicado por inúmeros estudos, cujos resultados de cálculos de energia produzem variações na faixa de 1,0 kcal.mol $^{-1}$ em relação aos dados experimentais. Os métodos semi-empíricos mais recentes são AM1 (*Austin Model 1*) [24] e PM3 (*Parametric Method 3*) [25] contidos em diversos pacotes de cálculos teóricos.

O método quântico semi-empírico AM1 (Austin Model 1) foi utilizado para calcular um conjunto de descritores moleculares para os compostos em estudo. Em seguida, os descritores foram analisados utilizando os métodos de reconhecimento padrão: Análise de Componentes Principais (PCA) [26] e Análise Hierárquica de Agrupamentos (HCA) [27].

Análise por Componentes Principais (PCA)

Esta técnica tem por objetivo a redução da dimensão dos dados originais facilitando a visualização das informações mais importantes em um número menor de componentes principais ou fatores.

Na PCA as coordenadas das amostras são reescritas num novo sistema de eixos (componentes principais), mais convenientes à análise dos dados. Neste novo sistema de eixos, cada componente principal é gerada a partir da combinação linear das m-variáveis originais, onde os componentes principais são ortogonais.

As combinações lineares das m-variáveis originais que dão origem a cada componente principal podem ser representadas por:

$$CP_i = a_{i1}v_1 + a_{i2}v_2 + \ldots + a_{im}v_m$$

Onde:

Vm = variáveis originais m= 1,2,...,m

a_{im} = *loading* ou peso, coeficiente que mede a importância de cada variável na i-ésima componente principal (PC_i).

Os *loadings* são o cosseno do ângulo entre o eixo da componente principal e o eixo da variável original. Logo, o seu valor estará entre –1 e 1. Quanto mais próximo de $\pm$ 1, maior a influência que esta determinada variável tem na descrição deste componente principal. Os *loadings* são capazes de determinar quais variáveis originais possuem maior contribuição na combinação linear de cada componente principal.

Análise por Agrupamentos Hierárquicos (HCA)

É o segundo método de análise exploratória, também chamada de técnica não supervisionada de reconhecimento de padrões. É uma técnica que tem como objetivo analisar a formação de agrupamentos naturais das amostras, com base nas suas similaridades. O primeiro passo é selecionar uma medida de similaridade. Em seguida, deve-se decidir o tipo de agrupamento hierárquico que será empregado e, finalmente, deve-se escolher o critério de ligação (*linkage*) entre os agrupamentos.

O agrupamento hierárquico mais comum é o aglomerativo, que funciona através de uma série de fusões. Inicialmente, todos os objetos estão separados e cada um é um pequeno grupo. A seguir, os dois objetos mais próximos (mais semelhantes) são unidos para formar um grupo de dois objetos. Se existe mais de um objeto com o mesmo grau de similaridade, o agrupamento é feito aleatoriamente. A união dos grupos (com um ou mais objetos) é feita sucessivamente até que todos os objetos estejam em um único grupo. Quando os grupos apresentam mais de um objeto, diferentes critérios de união podem ser usados.

O resultado de um agrupamento hierárquico é representado normalmente através de um gráfico bidimensional denominado dendrograma*,* onde é possível observar as correlações e similaridades entre as amostras.

A similaridade entre as amostras é avaliada medindo-se as distâncias entre os pares de amostras e colocando num mesmo agrupamento aquelas amostras com menores distâncias entre si. Em seguida, a distância entre esses pequenos agrupamentos é medida e são estabelecidos novos agrupamentos, assim por diante até que todas as amostras tenham sido enquadradas em algum grupo. Esse tipo de agrupamento permite observar os diferentes graus de similaridade entre as amostras, pois o agrupamento é feito de cada amostra individual em direção a um conjunto total.

Recentemente foi relatado na literatura [28] o estudo do fracionamento do extrato hexânico de *Myroxylon balsamum* (óleo vermelho), realizando em seguida, a partir destas frações, ensaio biológico larvicida (larvas de terceiro estádio de *A. aegypti*) para se determinar a(s) substância(s) ativa(s) contra a larva do mosquito transmissor do dengue. Os pesquisadores, além disso, para efeito de comparação, estudaram a atividade larvicida de vários terpenos e fenilpropanóides, constituintes de óleos essenciais abundantes encontrados em plantas brasileiras.

Baseado nos resultados de atividade biológica reportados pelos pesquisadores [28], o presente trabalho tem como objetivo estudar a relação estrutura-atividade de todas as substâncias ensaiadas, utilizando a Química Quântica e a Quimiometria.

Material e Métodos

Os descritores moleculares foram obtidos a partir de cálculos de Mecânica Molecular (MM+) [29] e Métodos Quânticos Semi-Empíricos AM1 [25], contidos nos pacotes de programas HyperChem 6.01 [30] e WinMopac 7.21 [31]. As propriedades calculadas foram correlacionadas com a atividade larvicida previamente conhecida a partir de dados da literatura [28].

Os descritores responsáveis pela atividade dos compostos foram separados por métodos quimiométricos. Os métodos utilizados foram a Análise por Componentes Principais (PCA) e Análise por Agrupamentos Hierárquicos (HCA), contidos no programa MINITAB 14® [32].

Resultados e Discussão

Pré-Modelagem das Moléculas Utilizando o Método Mecânica Molecular

As moléculas que apresentam atividade larvicida contra o mosquito *Aedes aegypti*, e as que não apresentam esta atividade [28] foram pré-otimizadas através de cálculos de Mecânica Molecular (MM+), contidos no pacote de programas HyperChem 6.01, com gradiente de convergência de 5 x 10^{-4} Kcal/mol. Com isso, foram obtidas as melhores conformações para essas moléculas, as quais foram posteriormente estudadas através de cálculos quânticos semi-empíricos. As moléculas estudadas estão mostradas na Figura 1.

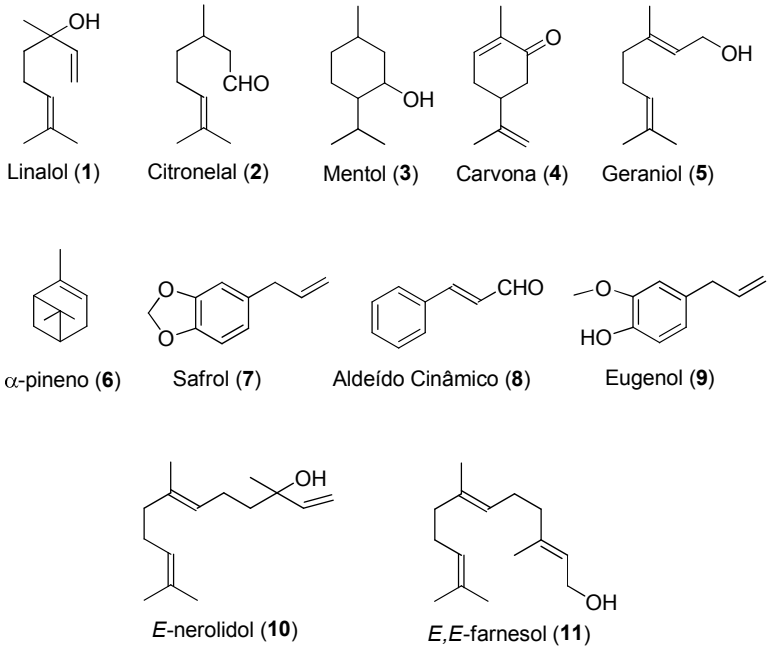

Figura 1. Estruturas das moléculas estudadas.

Obtenção dos Descritores Moleculares

As conformações moleculares das substâncias foram obtidas a partir dos cálculos de Mecânica Molecular, contidos do pacote de programas HyperChem 6.01, e foram aproveitadas como matrizes iniciais para a otimização das moléculas a partir de cálculos quânticos semi-empíricos AM1, contidos no pacote WinMopac 7.21.

As diversas aproximações semi-empíricas permitem evitar o cálculo de um grande número de integrais, o que possibilita a aplicação destes métodos em sistemas com um número maior de átomos. Nestes métodos, os núcleos são assumidos em sucessivas posições estacionárias, sobre as quais a distribuição espacial ótima dos elétrons é calculada pela resolução da equação de Schrödinger. O processo é repetido até que a energia não mais varie dentro de um limite escolhido, ou seja, até se alcançar um ponto estacionário de superfície de energia. Em um sistema no estado fundamental, isto significa que a geometria é tal que o calor de formação (ΔH_f) é um mínimo irredutível (na verdade um mínimo irredutível local), ou seja, todas as suas constantes de força são positivas; para estados de transição, o sistema deve ter exatamente uma constante de força negativa.

Propriedades moleculares de compostos químicos são geralmente correlacionadas com atividade biológica. Essa correlação é conhecida como *Relação Estrutura-Atividade* (SAR) e vários estudos têm sido reportados na literatura [33]. O método SAR tem sido usado com sucesso em aplicações farmacêuticas, e no presente trabalho foram calculadas as seguintes propriedades moleculares para serem correlacionadas com a atividade biológica em estudo: logP (propriedade relacionada com a lipofilicidade das substâncias): os valores desta propriedade foram obtidos a partir de parâmetros hidrofóbicos, usando o pacote de programas HyperChem 6.01; área superficial molecular, volume, energia de hidratação, refratividade e polarizabilidade também foram obtidas a partir de cálculos AM1 com o pacote de programas HyperChem 6.01. As demais propriedades eletrônicas (Energia Eletrônica, Energia Total, Momento de Dipolo, E_{HOMO}, E_{HOMO-1}, E_{HOMO-2}, E_{HOMO-3}, E_{LUMO}, E_{LUMO+1}, E_{LUMO+2}, E_{LUMO+3}) e os calores de formação foram obtidos a partir de cálculos AM1 com o pacote de programas WinMopac 7.21.

Em trabalho anterior [29], foi relatada a atividade larvicida para os compostos em estudo em *A. aegypti.* Foram determinadas as concentrações letais 50% (CL_{50}) e 99% (CL_{99}) a partir de ensaios biológicos segundo metodologia preconizada pela OMS (Tabela 1). Essas são as concentrações responsáveis pela erradicação de 50 e 99% das larvas presentes. Os valores de CL_{50} foram utilizados como base para a separação dos compostos em dois grupos: ativos e inativos.

Tabela 1. Susceptibilidade de larvas de terceiro estádio de *A. aegypti* a monoterpenos, sesquiterpenos e fenilpropanóides

Substâncias	**CL_{50} (ppm)**	**CL_{99} (ppm)**
Linalol* (**1**)	> 100	(-)
Citronelal* (**2**)	> 100	(-)
Mentol* (**3**)	> 100	(-)
Carvona* (**4**)	43.8 (39.0 – 48.3)	131.5 (101.5 – 214.0)
Geraniol* (**5**)	81.6 (77.5 – 86.7)	122.0 (111.6 – 139.0)
α-Pineno* (**6**)	74.3 (68.7 – 81.7)	168.1 (133.0 – 268.0)
Safrol** (**7**)	49.0 (47.5 – 50.2)	63.8 (59.7 – 73.2)
Aldeído Cinâmico** (**8**)	24.4 (22.4 – 26.5)	54.7 (45.1 – 76.8)
Eugenol** (**9**)	44.5 (40.8 – 48.0)	100.0 (83.0 –139.0)
Nerolidol*** (**10**)	17.0 (15.2 – 19.0)	50.0 (37.3 – 84.8)
Farnesol*** (**11**)	13.0 (12.0 – 14.1)	32.5 (25.5 – 52.5)

*monoterpeno, **fenilpropanóide, ***sesquiterpeno

Os parâmetros obtidos estão mostrados na Tabela 2 a seguir.

Antes de aplicar os métodos de reconhecimento padrão aos 11 compostos em estudo, foram verificadas as correlações entre as variáveis, segundo 4 critérios:

I – Correlação entre as variáveis calculadas para o composto **1** e do **4** ao **11**;

II - Correlação entre as variáveis calculadas para o composto **2** e do **4** ao **11**;

III - Correlação entre as variáveis calculadas para o composto **3** e do **4** ao **11**;

IV - Correlação entre as variáveis calculadas para todos os compostos.

Isto se deve ao fato de as atividades dos compostos **1**, **2** e **3** não serem exatamente conhecidas. O que se sabe é que a CL_{50} ultrapassa a faixa de 100 ppm para os três compostos. A melhor separação foi obtida utilizando–se os dados em I, ou seja, comparando-se as propriedades do composto inativo **1**, com os compostos **4** ao **11.**

Após análises dos dados por Peso de Fischer e Correlação entre as variáveis, a melhor separação dos compostos foi obtida utilizando-se as seguintes variáveis:

Tabela 2. Parâmetros obtidos a partir de cálculos quânticos semi-empíricos AM1

Composto	**Atividade (CL_{50} – ppm)**	**HOMO (EV)**	**HOMO – 1 (EV)**	**HOMO – 2 (EV)**	**HOMO – 3 (EV)**	**LUMO (EV)**	**LUMO + 1 (EV)**	**LUMO + 2 (EV)**	**LUMO +3 (EV)**	**M.D. (D)**
1	>100.00	-9.16	-10.05	-11.24	-11.31	1.26	1.33	3.34	3.55	1.41
2	>100.00	-9.39	-10.53	-11.47	-11.72	0.90	1.06	3.20	3.34	3.05
3	>100.00	-10.54	-10.75	-11.01	-11.19	3.34	3.58	3.69	3.75	1.40
4	43.80	-9.86	-10.04	-10.38	-11.79	-0.01	0.99	1.78	3.05	3.21
5	81.60	-9.31	-9.69	-10.91	-11.53	0.92	1.16	3.60	3.70	1.72
6	74.30	-9.12	-10.47	-10.89	-10.98	1.18	3.36	3.63	3.69	0.08
7	49.00	-8.86	-9.73	-10.18	-11.44	0.19	0.28	1.23	1.85	0.60
8	24.40	-9.34	-9.98	-10.66	-11.45	-0.72	0.28	0.74	1.96	3.04
9	44.50	-8.67	-9.47	-10.10	-11.48	0.35	0.46	1.33	2.62	1.73
10	17.00	-9.22	-9.26	-10.27	-10.94	1.15	1.20	1.29	3.35	1.41
11	13.00	-9.22	-9.34	-9.67	-11.10	0.89	1.18	1.20	3.56	1.75

Tabela 2. Continuação

Composto	Calor de Formação (Kcal/mol)	E.TOTAL (EV)	E.ELET (EV)	SURF. AREA APROX (Ă^2)	SURF. AREA GRID (Ă^2)	VOLUME (Ă^3)	E. HID. Kcal/mol	Log P	REFRAT. (Ă^3)	POLAR. (Ă^3)
1	-47.56	-1848.81	-10389.64	381.92	364.74	591.27	-2.18	2.52	50.21	19.38
2	-64.39	-1849.54	-10081.16	421.34	379.61	604.11	12.61	2.25	49.30	19.02
3	-97.86	-1878.31	-11368.48	359.36	369.64	588.89	-0.21	2.78	47.44	18.99
4	-27.95	-1793.34	-9606.66	343.60	352.38	549.07	0.73	2.55	47.17	18.05
5	-59.29	-1849.32	-10122.22	401.06	379.20	606.63	-2.64	2.46	51.18	19.38
6	16.79	-1500.03	-8892.57	303.07	334.99	519.86	2.49	2.80	44.72	17.38
7	-25.53	-2057.29	-10240.14	296.27	351.72	534.82	-5.70	2.78	46.11	18.08
8	5.50	-1581.72	-6990.81	304.58	316.74	474.18	13.39	1.74	42.13	15.83
9	-46.07	-2085.49	-10624.97	338.53	369.63	567.72	-7.68	2.55	48.50	18.86
10	-53.71	-2599.45	-17139.26	536.85	499.72	838.82	-1.22	4.00	74.01	28.36
11	-64.79	-2599.93	-17319.49	524.95	486.05	826.07	-3.07	3.94	74.98	28.36

E_{HOMO-1}, E_{HOMO-2}, E_{LUMO}, E_{LUMO+2}, LogP (parâmetro lipofílico). Os valores encontrados para estas variáveis estão apresentados na Tabela 2. Isto sugere que as demais variáveis calculadas não são significantes para a classificação dos compostos em ativos e inativos frente a larvas do mosquito *A. aegypti.*

Os resultados da Análise por Componentes Principais (PCA) mostram que a primeira componente (PC1) é responsável por 54,19% da variância dos dados. Considerando a primeira (PC1), a segunda (PC2) e a terceira (PC3) componentes, a variância acumulada alcança 97,00 %, como mostrado na Figura 2.

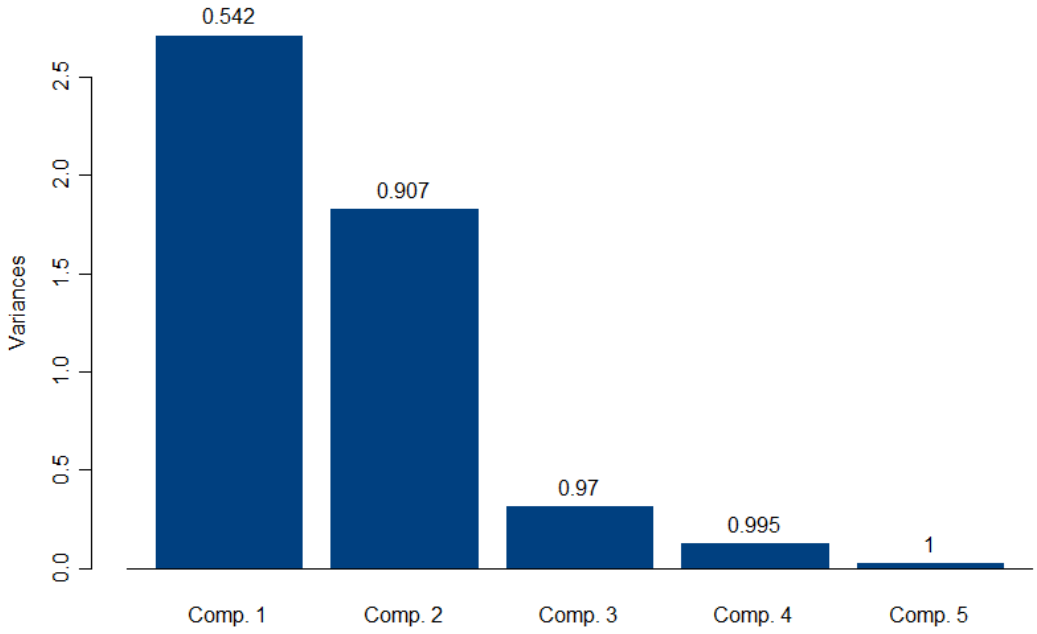

Figura 2. Importância relativa das componentes principais.

A Figura 3 (PC1 x PC2) mostra que a PC1 é fator responsável para a discriminação entre os compostos ativos (**4**, **7**, **8**, **9**, **10** e **11**) e inativos (**1**, **5** e **6**) sobre *A. aegypti,* quando utilizamos as variáveis E_{HOMO-1}, E_{HOMO-2}, E_{LUMO}, E_{LUMO+2} e LogP para obter a separação. A partir da Figura 3 podemos ver também que os compostos ativos apresentam valores de *scores* positivos para PC1, enquanto que os compostos inativos apresentam valores negativos.

A equação (1) apresenta os valores de *loading* (influência na componente principal) de cada variável em PC1, a qual é responsável pela discriminação entre os compostos ativos e inativos.

$$PC1 = 0.521\,[E_{HOMO-1}] + 0.570\,[E_{HOMO-2}] - 0.493\,[E_{LUMO+2}] + 0.388\,[\text{LogP}] \quad (1)$$

A equação (2) apresenta os valores *loading* de cada variável em PC2:

$$PC2 = -0.164\,[E_{HOMO-1}] - 0.727\,[E_{LUMO}] - 0.396\,[E_{LUMO+2}] - 0.534\,[\text{LogP}] \quad (2)$$

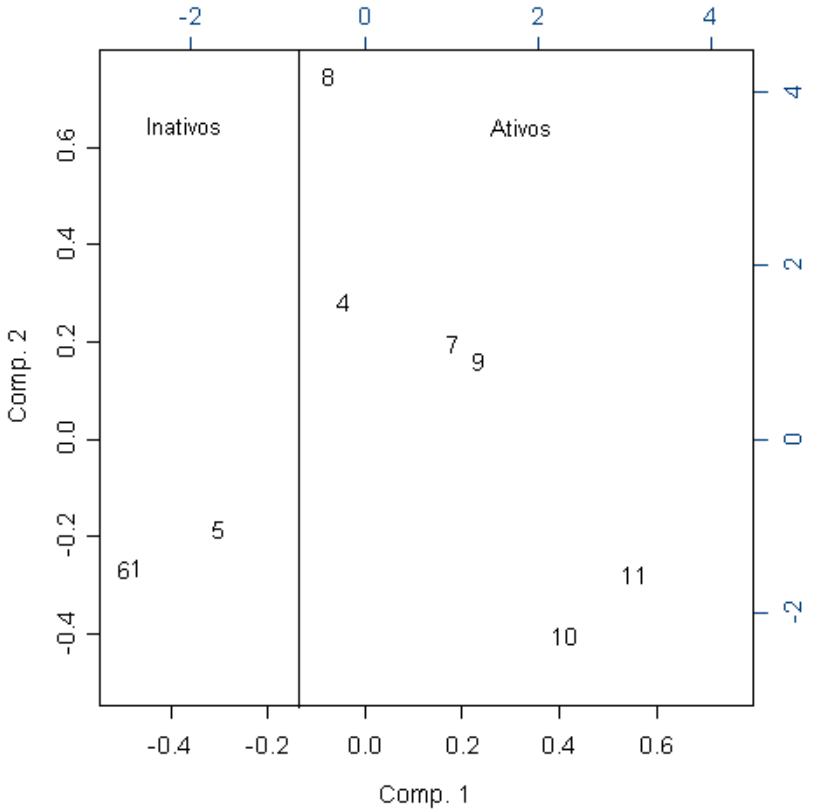

Figura 3. Separação dos compostos em dois grupos pelo método PCA: compostos ativos e inativos.

A partir da equação (1) podemos observar que para um composto ser classificado como ativo é necessário que este apresente os menores valores, em módulo, para a energia do HOMO-1, uma vez que todos são negativos. O mesmo é válido para a energia do HOMO-2. É necessário também que apresente valores altos e negativos para a energia do LUMO +2 e valores altos e positivos para o parâmetro lipofílico.

Isto é interessante porque podemos observar que as variáveis responsáveis pela separação dos compostos em ativos e inativos são propriedades eletrônicas (E_{HOMO-1}, E_{HOMO-2}, E_{LUMO}, E_{LUMO+2}) e apenas uma é estrutural (LogP). Com isso, podemos concluir que os efeitos eletrônicos mostrados, bem como a lipofilicidade, são de extrema importância para a determinação da atividade larvicida de compostos sobre larvas do mosquito *A. aegypti.*

As energias dos orbitais de fronteira são propriedades importantes em Química Farmacológica. A razão para isto está no fato de que estas propriedades nos fornecem informações a respeito do caráter doador e receptor de elétrons. A energia do orbital molecular de mais alta energia ocupado (HOMO) mostra o caráter doador de elétron e a energia do orbital molecular de mais baixa energia não-ocupado (LUMO) mostra o

caráter receptor de elétron. A partir destas definições, temos que: (1): altos valores para E_{HOMO} representam alta capacidade doadora de elétron e (2): baixos valores para E_{LUMO} representam baixa resistência à recepção de elétrons. Para os compostos ativos estudados, quando comparados aos inativos, menores valores para E_{LUMO}, o que mostra que os compostos ativos são receptores de elétrons mais eficientes que os inativos, o que nos sugere um possível mecanismo de transferência de cargas com o receptor biológico.

Os resultados obtidos a partir do HCA estão abrigados no dendrograma mostrado na Figura 4. A partir desse dendrograma, podemos classificar os compostos em dois grupos: compostos ativos (A), e inativos (B). Além disso, podemos observar a similaridade entre os compostos **11** e **12**, de maior atividade frente a larvas do mosquito *A. aegypti,* e a menor atividade do composto **1**.

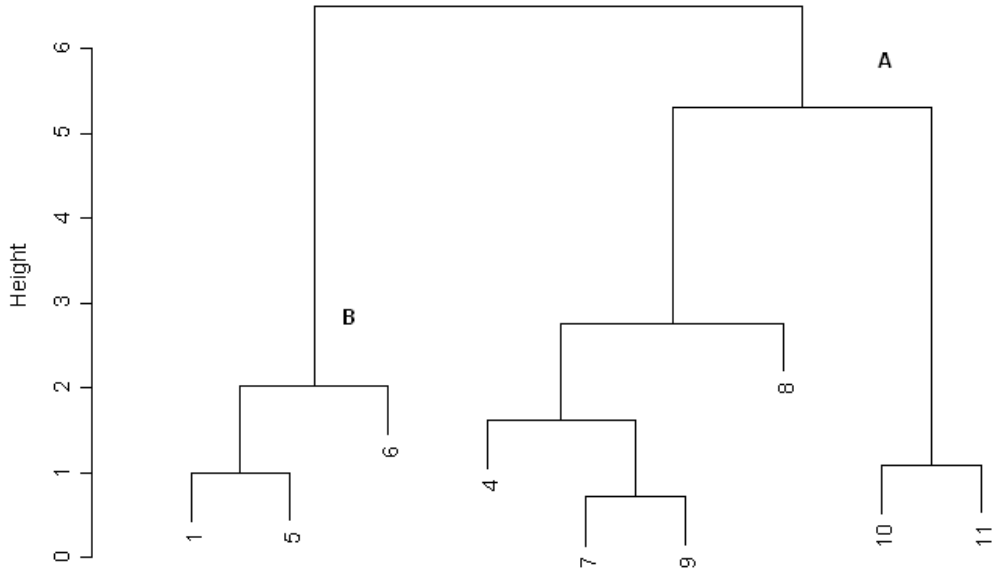

Figura 4. Dendrograma obtido com o método HCA, mostrando a separação dos compostos em dois grupos: A (compostos ativos) e B (compostos inativos).

Os métodos HCA e PCA são complementares e para os compostos estudados neste trabalho, foram bastante similares, classificando os compostos exatamente como esperado.

Conclusão

Os métodos de reconhecimento padrão (HCA e PCA) foram aplicados sobre os compostos com atividade larvicida sobre *A. aegypti* e mostraram que os compostos estudados neste trabalho puderam ser corretamente classificados em dois grupos:

moléculas ativas e inativas.

As variáveis eletrônicas E_{HOMO-1}, E_{HOMO-2}, E_{LUMO}, E_{LUMO+2} e a estrutural LogP, segundo os resultados de PCA são responsáveis pela separação entre compostos ativos e inativos. Os resultados obtidos a partir do HCA foram bastante similares àqueles obtidos por PCA, e ambos os métodos classificaram os compostos exatamente como esperado a partir dos dados experimentais.

A análise dos dados obtidos com os métodos quimiométricos (PCA e HCA), permitiu concluir que na maioria dos compostos, as variáveis eletrônicas são as responsáveis pela separação entre compostos ativos e inativos. Portanto, podemos concluir que os efeitos eletrônicos mostrados são de suma importância na separação dos compostos estudados quanto à atividade frente a larvas do mosquito *A. aegypti.*

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ABSTRACT

Use of chemometric and quantum-mechanical methods in the analysis of bioactive terpenoids and phenylpropanoids against the *Aedes aegypti*

Dengue fever is one of the main public health problems in the world. Many mosquitoes have developed resistance to the conventional insecticides used. Thus, the search for vegetable extracts and natural substances as alternative insecticides has increased. In this study, chemometric methods were employed to classify a group of terpenoid and phenylpropanoid compounds with biological activity against the larval of the A. aegypti mosquitoes. The AM1 (Austin Model 1) method was used to calculate a set of molecular descriptors (properties) for the studied compounds. Then, the descriptors were analyzed using the following methods of pattern recognition: Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA). The PCA and HCA methods have shown to be very effective for the classification of the study compounds in two groups (active and inactive). The electronic variables E_{HOMO-1}, E_{HOMO-2}, E_{LUMO}, E_{LUMO+2}, and the structural LogP were used to classify as active and inactive compounds. In most studied compounds, the variables responsible for separating active from inactive

compounds were electronic descriptors. Thus, it can be concluded that electronic effects play a fundamental role in the interaction between biological receptor and terpenoid and phenylpropanoid compounds with activity against larval A. aegypti mosquitoes.

Keywords: terpenes, dengue, AM1, chemometric, PCA

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Full Paper

Interaction of a di-nitro aniline herbicide (Trifluralin) with soil vegetation system under sub-tropic condition: A dissipation kinetics study

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ABSTRACT: *A field study was carried out to investigate persistence and dissipation kinetics of Trifluralin (48 EC) applied pre emergently in Green gram (Variety T-44) @ 1 Kg ai ha^{-1} (T_1) and 2 Kg ai ha^{-1} (T_2) for the control of broad leaf weeds during kharif 2006. The dissipation on 90 days was around 71.56 - 64.55% in T_1 and T_2. Kinetics studies revealed that dissipation of Trifluralin residues followed first order kinetics. The half life values observed were 60.21 days in T_1 and 75.56 days in T_2. Irrespective of any dose no residues were detected in cropped soil as well as plant samples at harvest.*

Keywords: *soil, green gram, residues, Trifluralin, dissipation, persistence*

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Introduction

Green gram, *Vigna radiata* (L.) R. Wilczek is one of the important summer pulse crops grown in India. It is affected by a number of broad leaf weeds like *Trianthema portulacastrum, Dactylocterium aegyptium, Echinochloa colonum* and *Digera arvensis*. The weeds compete with crop for nutrients, moisture, space and light and cause an average loss of about 25-50% depending upon the species and density of weeds [1, 2]. Yield losses due to weeds in Green gram have been reported to be 42-68% [3, 4].

Trifluralin - 2,6-dinitro-*N,N*-dipropyl-4-(trifluoromethyl)benzenamine - is a representative of dinitroaniline herbicide group and widely used against weeds of various crops like soybean, legumes, beans etc. It has been found effective for the control of broad leaf weeds of green gram [5]. It also controls many annual grasses in cotton, fruit trees, nut trees, vines, ornamentals, soya beans, groundnuts, oilseed rape, sunflowers, lucerne, peas, sweet potatoes, mint, and non-crop areas [6]. Though this herbicide controls the weed but they may persist in the soil. It may exist in the harvested pod or green plant also and thus cause health hazard to human being [7]. Studies on analytical methods for Trifluralin determination, metabolism in animals, plants and fish, degradation in soil, photodegradation, adsorption to soil, volatilization, persistence in fields, residues in agricultural crops and river water are briefly reviewed [8].

This crop is highly remunerative and sprayed heavily with herbicides close to harvest, which may leave harmful residues in consumable parts of the plants. Since there are no data available on the persistence of Trifluralin on green gram, the present investigation was conducted to determine the dissipation pattern as well as the residue level of the herbicide (Trifluralin) in plants under West Bengal (East Indian) climatic condition when applied @ 1.0 Kg.a.i.ha^{-1} (T_1) and 2.0 Kg.a.i.ha^{-1} (T_2) along with untreated control (T_3).

Material and Methods

Design of field experiment

Green gram (Variety T-44) crop was raised in fields of Agricultural Research Farm, Baruipur under the operational area of Institute of Agricultural Science, University of Calcutta, Kolkata, West Bengal, India in the month of July 2006. A randomized block design (RBD) with 3 replications and two treatments, including an untreated control was used. The plot size was 6 x 3 m.

Climatic conditions

The climatic parameters for the season (July, 2006 - November, 2006) were: temp. min. 22.13 °C, max. 31.42 ^{o}C; relative humidity 93.65%; rain fall 258 mm.

Application of herbicides

Trifluralin (Treflan 48 EC) was applied @ 1 Kg ai ha^{-1} (T_1 recommended dose) and 2 Kg ai ha^{-1} (T_2, double the recommended dose) as a pre emergent herbicide. Identical portions of plants were maintained as controls and were sprayed with water.

Collection of samples

Representative samples (1 Kg) of soil from 0-15 cm depth were collected from 10 places using a steel auger tube. The samples were collected at 0 (2 h), 3, 7, 15, 30, 60 and 90 days after treatment. Plant samples (seeds or pods) along with cropped soil samples were collected only at harvest (120 days after application).

Extraction and clean up of residues

The soil samples were dried under shade, ground, sieved through 2 mm mesh sieve and 20 g representative sample was taken in a 250 mL conical flask. Two drops (0.5 mL) of ammonia solution was added to the flask and was thoroughly mixed and then left till there was no smell of ammonia.

The samples were extracted and cleaned-up as per following method. Soil samples were extracted with 100 mL acidic methanol (98% methanol, 2% concentrated HCl) by shaking for 1.5 h on a mechanical shaker. The extracts were centrifuged at 2000 rpm for 10 min and he supernatant transferred to 1 L separator funnels. Extracts were subjected to liquid-liquid partitioning with hexane thrice (50, 30, 20 mL) after diluting with 250 mL solution of 10% sodium chloride. The hexane extract was concentrated to 10 mL on rotary vacuum evaporator after addition of one drop of mineral oil.

Glass column (60 cm x 22 mm id) was compactly packed with 4.5 g adsorbent mixture (Florisil: Celite:activated charcoal 2:2:0.5) in between the layers of anhydrous sodium sulphate. The column was prewetted with hexane and concentrated extract loaded at the top. The column was eluted with 125 mL mixture of benzene: hexane (3:2). The eluent was concentrated first on rotary vacuum evaporator followed by gas manifold evaporator. The final volume was made to 2 mL with freshly distilled *n*-hexane.

Estimation of residues

The samples were analyzed on Hewlett Packard 6890N Network Series coupled with autosampler gas chromatograph equipped with ^{63}Ni electron capture detector (ECD) and SPB-5 capillary column (30 m x 0.32 mm Id, 0.25 m film thickness). The GC parameters were as follows: Temperatures - column 180°C, injector port 250°C and detector 250°C; flow of carrier gas (N_2) 2 mL/min, split ratio 1:10. Retention time

observed for Trifluralin was 3.31 min. The limit of detection (LOD) and limit of quantification (LOQ) were 0.01 µg g^{-1} and 0.03 µg g^{-1}, respectively.

Recovery experiment

In order to estimate the efficiency of the method, recovery experiment was conducted by fortifying untreated samples of green gram seeds or pods and cropped soil with Trifluralin (analytical standard, purity 98.97%, Sigma Aldrich) @ 0.25, 0.50 and 1.00 µg g^{-1} level. The fortified samples were analyzed and estimated following the method as described earlier.

Results and Discussion

Average recoveries of Trifluralin from different substrates fortified @ 0.25, 0.50 and 1.00 µg g^{-1} ranged from 90-96% and 88-98% for cropped soil and green gram seeds or pods, respectively (Table 1).

Table 1. Results of method validation by recovery analysis of Trifluralin (analytical grade) from test samples.

Substrates	Amount fortified (µg g^{-1})	Amount recovered (µg g^{-1})	Recovery of Propineb (%)	Avarage recovery of Propineb (%)
Cropped Soil	0.25	0.225	90	93.33
	0.50	0.47	94	
	1.00	0.96	96	
Green gram seeds or pods	0.25	0.22	88	94.00
	0.50	0.48	96	
	1.00	0.98	98	

The residue data is presented in Table 2. The initial residues were 1.778 and 3.179 µg g^{-1} in T_1 and T_2 which dissipated to 1.664 and 2.876 µg g^{-1} only in 3 days recording a mere loss of 6.38 and 9.54%, respectively. The residues persisted beyond 90 days. On 90^{th} day, 71.56 and 64.55% dissipation was recorded. The residue data was subjected to analysis using Hoskin's method [9]. It is clear from the Figure 1 that Trifluralin exhibits first order dissipation. The dissipation was slow, probably because of its high affinity towards soil organic matter (Koc = 4400). No residue was detected in seeds as well as in cropped soil samples at harvest (120 days after application) irrespective of any dose (Table 3) indicating no translocation of the herbicide from soil to

different plant parts. The half-life values for single and double doses were 60.21d and 75.26d respectively indicating slightly slower dissipation at high rate of application (Table 4).

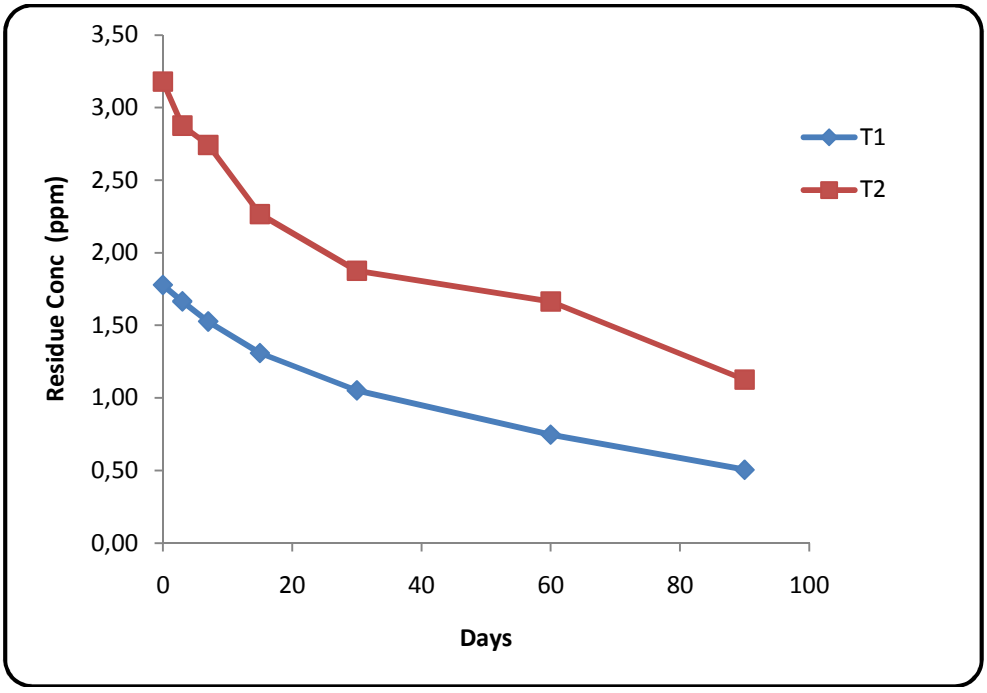

Figure 1. Dissipation of Trifluralin under green gram cropped soil.

Table 2. Persistence and dissipation of Trifluralin residues in cropped soil.

Sampling Interval (in days)	Residues (in µg g^{-1}) M*± SD (% of Dissipation)	
	T_1 (1.0 kg a.i. ha^{-1})	T_2 (2.0 kg a.i. ha^{-1})
0	1.778±0.173 (-)	3.179±0.743 (-)
3	1.664±0.097 (6.38)	2.876±0.106 (9.54)
7	1.526±0.069 (14.16)	2.742±0.476 (13.73)
15	1.309±0.030 (26.37)	2.267±0.017 (28.70)
30	1.052±0.066 (40.85)	1.876±0.021 (40.99)
60	0.748±0.036 (57.94)	1.664±0.540 (47.66)
90	0.506±0.004 (71.56)	1.127±0.005 (64.55)

M* = Mean of three replicate

As evident from the data, the dissipation of Trifluralin was slow (64.55-71.56% on 90d) under the field condition. Thus chemistry of Trifluralin after soil application was preferably governed by the adsorption phenomenon. The physicochemical as well as biological transformation processes such as volatilization and leaching loss, runoff, microbial degradation, hydrolysis etc. operates at a slow pace. In present study, results regarding uniphasic dissipation of Trifluralin are not in confirmatory to earlier reports

[10-12]. In another report, half- life period in fresh clay soil has been reported to be 94-99 day [13] which is similar to our observation. The differences could be due to depth of incorporation, soil temperature, availability of soil moisture, soil aeration and soil organic matter content. Quite similar to our results, half-life period of 27 and 30 days in vineyard soil has been reported from South Australia [14].

Table 3. Residue of Trifluralin in green gram seeds and cropped soil at harvest.

Days after application	Substrates	Treatment	Residues in ppm (μg/g) (M* ± S.D.) [Dissipation (%)]	
			Season-I	Season-II
Harvest	Seeds	T_1 (1.0 kg a.i. ha^{-1})	BDL [-]	BDL [-]
	Soil		BDL [-]	BDL [-]
Harvest	Seeds	T_2 (2.0 kg a.i. ha^{-1})	BDL [-]	BDL [-]
	Soil		BDL [-]	BDL [-]

BDL = Below detectable limit (<0.01 ppm)
M* = Mean of three replicate

Table 4. Regression equations for first order dissipation of Trifluralin.

Treatments	Regression equation	Correlation coefficient (r)	Half life (days)
1.0 Kg ai ha^{-1}	Y = 3.226-0.005 X	0.991	60.21
2.0 Kg ai ha^{-1}	Y = 3.462-0.004 X	0.964	75.26

Conclusion

The dissipation of Trifluralin in soil under green gram in field conditions was quite fast. Although the residues remained in detectable amount beyond 90 days but the residues at these levels may not affect the next crop in rotation. As the residues were found below the detectable limit in all harvest samples (120 days after application), therefore it might be stated that Trifluralin may not cause any residual toxicity problem in green gram which is also befitting with the harvest schedule of green gram cultivation of eastern region of our country.

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Full Paper

Synthesis and studies of bissydnone sulfonamides based on 4,4'-diaminodiphenyl methane

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ABSTRACT: *3,3'-(4,4'-Diphenyl)bissydnonyl methane* ***(5)*** *was synthesized and subjected to chlorosulfonation followed by amination resulted in to formation of 3,3'-(methylenedi-1,4-phenylene)bis[4-{(4-substituted-amino)sulfonyl} sydnone* ***(7a-j)****. The structure of the newly synthesized compounds was checked by spectral data and purity of the compounds was checked by elemental analysis as well as thin layer chromatography. Some compounds showed excellent activity against gram positive and gram negative bacterial strain.*

Keywords*: sulfonamide, mesoionic, sydnone, NMR spectroscopy, IR spectroscopy, elemental analysis*

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Introduction

During past years many interesting data have been obtained on the structures [1, 2], reactivities [3, 4], physicochemical properties [5, 6] and biological actions [7-10] of sydnone compounds. In recent years much attention has been devoted to the synthesis of sydnone and their biological activities. Carbon atom at 4th position of sydnone ring bears a partial negative charge, accordingly electrophilic substitution for instance halogenation [11], nitration [12], acylation [13], and sulfonation [14] occurs at 4th position.

Sydnone derivatives derived from 4,4'-bis(aminophenyl)sulfone (Figure 1) were found to possess antimalarial activity [15].

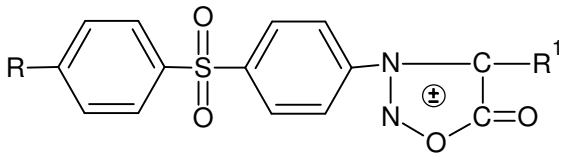


Figure 1. 4,4'-bis(aminophenyl)sulfones. Where R=Cl, NO_2 and R^1= H, Br.

3,3'-(4,4'-Diphenyl)bissydnonyl methane (Figure 2) showed strong effect on coronary dilation and inhibition of platelet aggregation. 3,3'-(4,4'-Diphenyl)bissydnonyl ether also showed cardiotropic and inhibition of platelet aggregation [16].

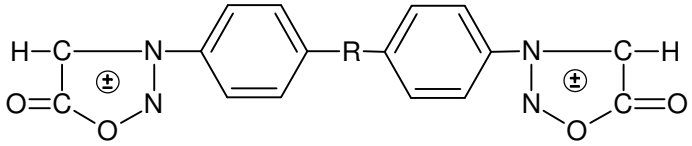


Figure 2. 3,3'-(4,4'-Diphenyl)bissydnonyl methane (R = CH_2) and 3,3'-(4,4'-Diphenyl)bissydnonyl ether (R = O).

Sulfonamide derivatives have proved fruitful area of research and subject of much interest due to their importance for various applications, and their widespread potential and proven biological and pharmacological activities. Sydnones have played a crucial role in the development of theory in heterocyclic chemistry and occupy a unique place in heterocycles. Badami et al [17-19] have synthesized various 3-arylsydnone-4-sulfonamide derivatives having potent biological properties.

These observations encouraged us to continue our current work to synthesize new

heterocyclic containing bissydnone sulfonamides based on 4,4′-diamino diphenyl methane.

Material and Methods

General

The starting material 4,4′-diaminodiphenyl methane was received from Atul Limited, Valsad. All other the reagents were of AR grade. All the melting points were uncorrected and were determined by open tube capillary method. CHN analysis was carried Carlo Erba 1108. IR spectra (KBr) were recorded on Shimadzu, Japan FTIR spectrophotometer. ^{1}H NMR spectra were recorded on Bruker Advance II 400 MHz NMR spectrometer and ^{13}C NMR spectra were recorded on Bruker Advance II 40 MHz NMR spectrometer using deuterated dimethylsulphoxide (DMSO-d_6) as a solvent and TMS as an internal standard. Purity of the compounds was checked by TLC on silica gel plates.

Chemistry

*2,2′-[Methylenebis(1,4-phenyleneimino)]diethyl acetate (**2**)*

4,4′-Diaminodiphenyl methane (1.98 g, 0.01 mol), ethylchloroacetate (2.13 mL, 0.02 mol) in dry ethanol (10 mL) and anhydrous sodium acetate (3.28 g, 0.04 mol) were refluxed for 5 hours. The mixture was diluted with water (10 mL). After standing overnight in the refrigerator, crystalline ester was obtained. The crude solid was purified by recrystallization from ethanol. Yield: 3.01 g, 80 %., m.p. 110-112 ^{0}C. I.R. (KBr): 2959, 2928, 2875, 2854, 1756, 1521,1307 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.34 (t, 6H, CH_3), 3.95 (s, 2H, NH), 4.05 (s, 6H, CH_2), 4.35 (q, 4H, OCH_2), 6.50-7.00 (m, 8H, Ar-H),. ^{13}C NMR (40 MHz, DMSO-d_6): δ 14.64, 40.50, 44.26, 61.24, 112.65, 128.95, 129.01, 145.87,170.97. Anal (%) for $C_{21}H_{26}N_2O_4$, Calcd. C, 68.09; H, 7.07; N, 7.56. Found: C, 68.13; H, 7.11; N, 7.58.

*2,2'-[Methylenebis(1,4-phenyleneimino)]diacetic acid (**3**)*

Compound **2** (3.70 g, 0.01 mol) and sodium hydroxide (1.2 g, 0.03 mol) were dissolved in a solution of distilled water and ehanol (36:4mL). The mixture was stirred at reflux temperature for 30 minutes. The resultant mixture was cooled and acidified with hydrochloric acid. White crystalline product was obtained, it was recrystallized from ethanol. Yield: 2.23 g, 70 %, m.p. 130-135 ^{0}C. I.R. (KBr): 2925, 2856, 2570-3209, 1719, 1518,1297 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.05 (s, 2H, CH_2), 4.10 (s, 4H, CH_2), 6.35 (s, 2H, NH), 6.40 (s, 2H, OH), 6.50-7.10 (m, 8H, Ar-H). ^{13}C NMR (40 MHz, DMSO-d_6): δ 40.82, 44.75, 112.28, 129.22, 129.47, 145.63, 171.54. Anal (%) for

$C_{17}H_{18}N_2O_4$. Calcd. C, 64.96; H, 5.77; N, 8.91. Found: C, 64.94; H, 5.79; N, 8.96.

*2,2'-{Methylenebis[1,4 -phenylene (nitrosoimino)]}diacetic acid (**4**)*[20]

To an ice-cold and well stirred solution of compound **3** (5.02 g, 0.016 mol) in water (40 mL), a freshly prepared sodium nitrate solution (3.32 g, 0.049 mol) was added drop wise over a period of 40 minutes. Concentrated hydrochloric acid was added till complete precipitation and allowed to stir cold solution for several minutes. The solid nitroso compound was filtered off and washed with cold water and dried. Yield: 3.80 g, 64 %. m.p. 130-135 ^{0}C. I.R. (KBr): 2925, 2853, 2570-3200, 1719, 1554, 1325 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.12 (s, 2H, CH_2), 4.84 (s, 2H, N-CH_2), 7.25-7.54 (m, 8H, Ar-H), 11.36 (s, 2H, COOH). ^{13}C NMR (40 MHz, DMSO-d_6): δ 40.61, 48.94, 122.88, 129.54, 137.20, 138.63, 167.61. Anal (%) for $C_{17}H_{16}N_4O_6$ Calcd. C, 54.84; H, 4.33; N, 15.05. Found: C, 54.81; H, 4.37; N, 14.98.

*3,3'-(4,4'-Diphenyl)bissydnonyl methane (**5**)*

The dried compound **4** (4.00 g, 0.0107 mol) was stirred for 12 hours in 40 mL acetic anhydride. The solution was poured slowly into cold water which was very well stirred. The pH of the content was adjusted to 7.0 with 10 % sodium bicarbonate solution. The solid crude product was washed well with water and dried. The crude sydnone was recrystallized from benzene-petroleum ether. The product obtained was orange solid.Yield: 2.52 g, 70 %. m.p. 120-123 ^{0}C. I.R. (KBr): 3157, 2925, 2853 1745 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.26 (s, 2H, CH_2), 7.76 (s, 2H, sydnone), 7.14-8.13 (m, 8H, Ar-H). ^{13}C NMR (40 MHz, DMSO-d_6): δ 39.43, 121.89, 128.26, 129.20, 139.36, 141.15, 168.20. Anal (%) for $C_{17}H_{12}N_4O_4$. Calcd. C, 60.71; H, 3.60; N, 16.66. Found: C, 60.68; H, 3.65; N, 16.63.

*3,3'-(Methylenedi-1,4-phenylene)bis(4-chlorosulfonyl) sydnone (**6**)*

Chlorosulfonic acid (2.32 mL, 0.02 mol) was added drop wise into the mixture of compound **5** (3.36 g, 0.01 mol) and catalytic amount of P_2O_5 over 30 minutes with constant stirring at 0-5 ^{0}C. The temperature of the well-stirred mixture does not rise above 5 ^{0}C. When all the chlorosulphonic acid has been added, reflux the mixture at about 60 ^{0}C for about 1 hour. The solution was then poured into a mixture of crushed ice and water with vigorous stirring. Precipitation was collected by filtration, washed three times with water and dried. Yield: 3.94 g, 74 %. m.p. 239-241 ^{0}C. I.R. (KBr): 2928, 1745, 1395, 1180 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.25 (s, 2H, CH_2), 7.29-8.12 (m, 8H, Ar-H). ^{13}C NMR (40 MHz, DMSO-d_6): δ 39.65, 121.76, 122.97, 128.58, 138.86, 140.85, 169.64. Anal (%) for $C_{17}H_{10}Cl_2N_4O_8S_2$. Calcd. C, 38.29; H, 1.89; N, 10.51. Found: C, 38.12; H, 2.01; N, 10.43.

General procedure for the synthesis of 3,3'-(Methylenedi-1,4-phenylene)bis[4-{(4-

*substituted-amino)sulfonyl} sydnone (**7a-j**)*

3,3'-(Methylenedi-1,4-phenylene)bis(4-chlorosulfonyl)sydnone (**6)** (5.86 g, 0.011 mol) was dissolved in acetone at room temperature. A solution of amine (0.022 mol) in acetone was added drop wise in to the solution of 3,3'-(Methylenedi-1,4-phenylene)bis(4-chlorosulfonyl)sydnone (**6)** over a period of 5 hours with constant stirring. Add 1.0 mL of pyridine to the well stirred solution after 1 hour and 2 hour respectively during the reaction. The solution was poured in to ice with stirring. Precipitation was collected by filtration, washed thrice with water and dried. Recrystallization from benzene.

*I.R., ^{1}H NMR and ^{13}C NMR of newly synthesized compounds **(7a-j)***

*3,3'-(Methylenedi-1,4-phenylene)bis[4-{(4-methylpiperazin-1-yl) sulfonyl} sydnone] (**7a**)*

Yield: 4.64 g, 64 %., m.p. 187-189 ^{0}C. IR (KBr): 1143, 1370, 1737, 2869, 2930, 2967, 3378 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.28-8.22 (m, 8H, Ar-H), 4.28(s, 2H, CH_2), 3.66 (t, 4H, SO_2NCH_2), 2.75 (t, 4H, NCH_2), 2.46 (s, 6H, CH_3); ^{13}C NMR (40 MHz, DMSO-d_6): δ 160.86, 141.76, 129.43, 128.96, 121.34, 89.76, 52.59, 44.50, 41.23, 39.53. Anal.(%) for $C_{27}H_{32}N_8O_8S_2$. Calcd. C, 49.08; H, 4.88; N, 16.96. Found: C, 49.12; H, 4.85; N, 16.93.

*3,3'-(Methylenedi-1,4-phenylene)bis[4-{(4-morpholin-4-yl)sulfonyl} sydnone] (**7b**)*

Yield: 4.19 g, 60 %., m.p. 170-172 ^{0}C. IR (KBr): 1143, 1158, 1372, 1734, 2933 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.23-8.18 (m, 8H, Ar-H), 4.27 (s, 2H, CH_2), 3.47-3.73 (m, 16H, morpholine); ^{13}C NMR (40 MHz, DMSO-d_6): δ 160.49, 141.62, 129.85, 129.11, 121.17, 89.70, 62.72, 42.56, 39.30. Anal.(%) for $C_{25}H_{26}N_6O_{10}S_2$. Calcd. C, 47.31; H, 4.13; N, 13.24. Found: C, 47.36; H, 4.05; N, 13.29.

*3,3'-(Methylenedi-1,4-phenylene)bis[4-{(diethylamino)sulfonyl} sydnone] (**7c**)*

Yield: 4.26 g, 64 %, m.p. 178-180 ^{0}C. IR (KBr): 954, 1143, 1375, 1735, 2931, 2884, 2849 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.23-8.18 (m, 8H, Ar-H), 1.18 (t, 12H, CH_3), 3.15 (q, 8H, CH_2), 4.23 (s, 2H, CH_2); ^{13}C NMR (40 MHz, DMSO-d_6): δ 160.96, 141.24, 129.92, 129.10, 121.23, 89.36, 42.15, 39.54, 14.96. Anal.(%) for $C_{25}H_{30}N_6O_8S_2$. Calcd. C, 49.49; H, 4.98; N, 13.85. Found: C, 49.41; H, 4.95; N, 13.79.

*3,3'-(Methylenedi-1,4-phenylene)bis[4-{(diphenylamino)sulfonyl} sydnone] (**7d**)*

Yield: 5.01 g, 57 %, m.p. 173-175 ^{0}C. IR (KBr): 1148, 1364, 1742, 2936 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.32-8.38 (m, 28H, Ar-H), 4.23 (s, 2H, CH_2); ^{13}C NMR (40 MHz, DMSO-d_6): δ 160.59, 142.69, 141.52, 130.32, 129.91, 129.15, 128.95, 127.73, 121.54, 89.43. Anal. (%) for $C_{41}H_{30}N_6O_8S_2$. Calcd. C, 61.64; H, 3.79; N, 10.52. Found: C,

61.69; H, 3.85; N, 10.59.

*3,3'-(Methylenedi-1,4-phenylene)bis[4-{(4-phenylpiperazin-1-yl) sulfonyl} sydnone] (**7e**)*

Yield: 5.10 g, 59 %, m.p. 184-186 ^{0}C. IR (KBr): 1145, 1372, 1738, 2930, 3372 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.80-8.33 (m, 18H, Ar-H), 4.31 (s, 2H, CH_2), 3.75-3.68 (m, 16H, piperazine); ^{13}C NMR (40 MHz, DMSO-d_6): δ 160.64, 152.54, 141.63, 129.70, 129.15, 129.04, 121.08, 119.86, 116.45, 89.71, 50.55, 44.48, 39.30. Anal.(%) for $C_{37}H_{36}N_8O_8S_2$. Calcd. C, 56.62; H, 4.62; N, 14.28. Found: C, 56.68; H, 4.68; N, 14.33.

*3,3'-(Methylenedi-1,4-phenylene)bis[4-{(imidazol-1-yl)sulfonyl} sydnone] (**7f**)*

Yield: 4.06 g, 62 %, m.p. 171-173 ^{0}C. IR (KBr): 1151, 1365, 1740, 2937, 3157-2830 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.35 (s, 2H, N=CH-N), 7.31-8.42 (m, 8H, Ar-H), 8.32 (d, 2H, NCH), 7.75 (d, 2H, NCH), 4.25 (s, 2H, CH_2); ^{13}C NMR (40 MHz, DMSO-d_6): δ 160.75, 141.52, 138.12, 135.73, 121.80, 129.78, 129.21, 119.78, 89.55, 39.30 Anal.(%) for $C_{23}H_{16}N_8O_8S_2$. Calcd. C, 46.31; H, 2.70; N, 18.78. Found: C, 46.36; H, 2.64; N, 18.84.

*3,3'-(Methylenedi-1,4-phenylene)bis[4-{(4-ethylpiperazin-1-yl)sulfonyl} sydnone] (**7g**)*

Yield: 4.39 g, 58 %, m.p. 166-168 ^{0}C. IR (KBr): 1145, 1363, 1743, 2864, 2879, 2932, 2968, 3374 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.31-8.27 (m, 8H, Ar-H), 4.25 (s, 2H, CH_2), 2.92 (t, 8H, N-CH_2), 2.73 (q, 4H, CH_2), 2.60 (t, 8H, SO_2NCH_2), 1.25 (t, 6H, CH_3); ^{13}C NMR (40 MHz, DMSO-d_6): δ 160.54, 141.43, 129.95, 129.13, 121.19, 89.37, 51.10, 50.64, 40.23, 39.32, 11.12. Anal. (%) for $C_{29}H_{36}N_8O_8S_2$. Calcd. C, 50.57; H, 5.27; N, 16.27. Found: C, 50.52; H, 5.23; N, 16.33.

*3,3'-(Methylenedi-1,4-phenylene)bis[4-{(4-[2,6-dichlorophenyl]piperazin-1-yl) sulfonyl}sydnone] (**7h**)*

Yield: 6.59 g, 65 %, m.p. 169-171 ^{0}C. IR (KBr): 1092, 1146, 1361, 1744, 2933, 3381 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.19-8.25 (m, 14H, Ar-H), 4.25 (s, 2H, CH_2), 3.87-3.79 (m, 16H, piperazine); ^{13}C NMR (40 MHz, DMSO-d_6): δ 160.64, 143.88, 136.65, 129.97, 129.11, 128.62, 125.75, 121.32, 89.66, 50.21, 44.62, 39.30. Anal.(%) for $C_{37}H_{32}Cl_4N_8O_8S_2$. Calcd. C, 48.17; H, 3.50; N, 12.14. Found: C, 48.23; H, 3.54; N, 12.18.

*3,3'-(Methylenedi-1,4-phenylene)bis[4-{(2-methylpiperazin-1-yl)sulfonyl} sydnone] (**7i**)*

Yield: 3.99 g, 55 %, m.p. 158-160 ^{0}C. IR (KBr): 1157, 1367, 1729, 2878, 2945, 2963, 3378 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.30-8.22 (m, 8H, Ar-H), 4.53 (q, 2H, N-CH piperazine), 4.26 (s, 2H, CH_2), 3.67 (t, 4H, NCH_2 piperazine), 3.21 (t, 4H, CH_2

piperazine), 2.80 (d, 4H, CH_2 piperazine), 1.78 (s, 2H, NH), 1.39 (d, 6H, CH_3); ^{13}C NMR (40 MHz, DMSO-d_6): δ 160.34, 141.67, 129.67, 128.54, 121.32, 89.86, 50.94, 47.32, 45.70, 41.64, 39.26, 17.44. Anal.(%) for $C_{27}H_{32}N_8O_8S_2$. Calcd. C, 49.08; H, 4.88; N, 16.96. Found: C, 49.12; H, 4.94; N, 16.93.

*3,3'-(Methylenedi-1,4-phenylene)bis[4-{(piperazin-1-yl)sulfonyl} sydnone] (**7j**)*

Yield: 5.01 g, 57 %, m.p. 163-165 ^{0}C. IR (KBr): 1163, 1364, 1737, 2945, 3370 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ 7.25-8.17 (m, 8H, Ar-H), 4.28 (s, 2H, CH_2), 3.67-3.26 (m, 16H, $NHCH_2$), 2.80 (s, 2H, NH); ^{13}C NMR (40 MHz, DMSO-d_6): δ 160.64, 141.56, 129.89, 129.11, 121.12, 89.46, 45.12, 41.81, 39.30. Anal. (%) for $C_{25}H_{28}N_8O_8S_2$. Calcd. C, 47.46;H, 4.46; N, 17.71. Found: C, 47.54; H, 4.53; N, 17.65.

Antibacterial Activity

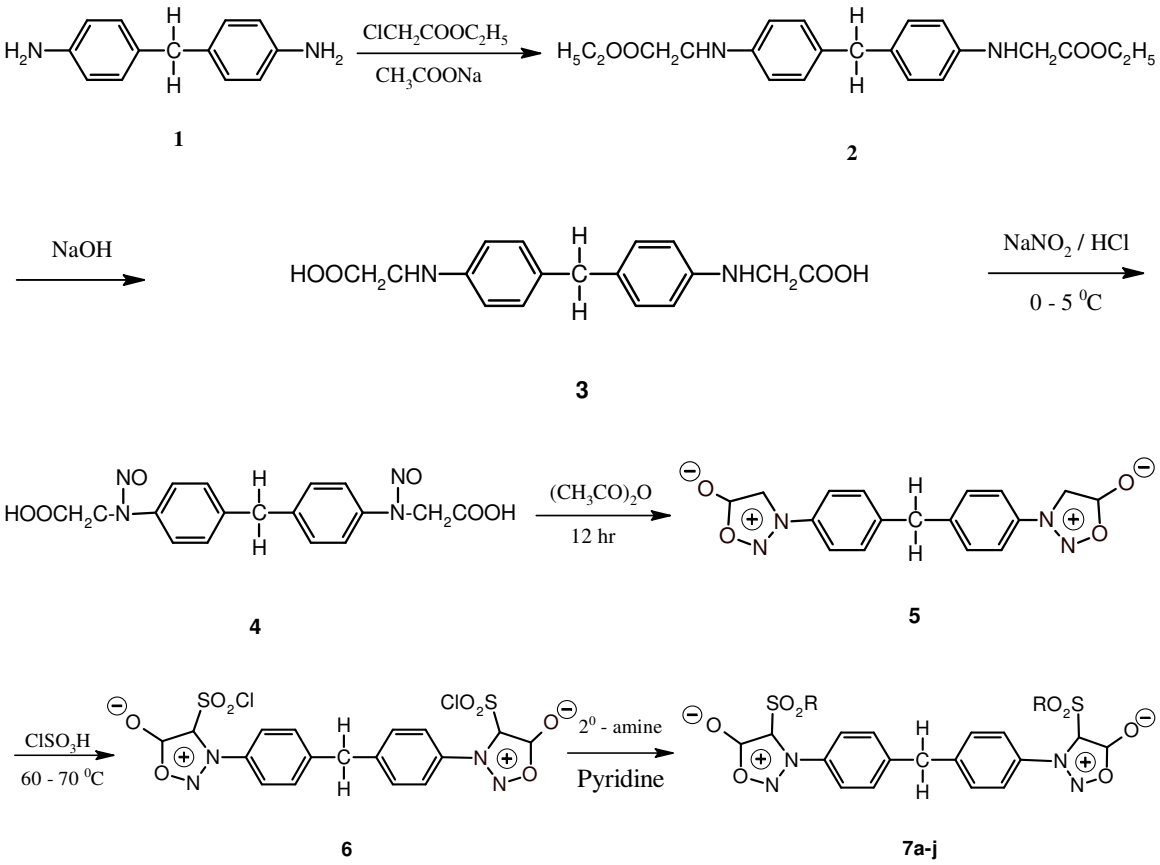
Newly synthesized compounds were also screened for their antibacterial activity against four species of bacterial strain in which two are gram positive bacteria, they are *Streptococcus pneumoniae*, *Staphylococcus aureus* and rest of two are gram negative bacteria, they are *Escherichia coli*, *Pseudomonas aeruginosa* and compare with standard drugs Penicillin and Streptomycin. Minimum Inhibitory Concentration (MIC) was determined by Broth Dilution Method [21] and Zone of Inhibition was determined by Agar Cup Method (Kirby-Bauer Technique) [22].

The sample compounds were screened at 200 µg/mL under identical conditions and the zone of inhibition was measured in mm. A reading of 10 mm indicates no zone.

Results and Discussion

Herein we have described the synthesis, characterization and biological evaluation of novel 3,3'-(methylenedi-1,4-phenylene)bis(4-substituted amino sulfonyl)sydnone (**7a-j**). The reaction of 3,3'-(methylenedi-1,4-phenylene)bis(4-chlorosulfonyl)sydnone (**6**) with substituted amine derivatives led to the formation of 3,3'-(Methylenedi-1,4-phenylene)bis[4-{(substituted amino)sulfonyl}sydnone (**7a-j,** Scheme 1). All compounds were analyzed satisfactorily by CHN elemental analysis. The I.R spectrum of the compounds displayed absorption band between 1729-1744 cm^{-1} which is the characteristics of carbonyl group of sydnone and also showed 1361-1375, 1143-1157 and 2930-2945 cm^{-1}, characteristic of $SO_{2asy,}$ SO_{2sym} and CH_2 group. All the IR spectral characteristics of different sulfonamide samples are in good agreement with proposed structure and are shown in experimental section. The IR spectrum of compound **5** showed absorption bands at 1745 cm^{-1} characteristic to the C=O group. The ^1H NMR

spectra of compound **5** showed a singlet at δ 7.76 ppm characteristic to the proton at C_4 of the sydnone. The ^{1}H NMR of compounds (**7a-j**) displayed the chemical shift value near δ 7.19-8.42 ppm is due to the presence of aromatic protons. ^{13}C NMR of carbon at four position of sydnone ring resonance at near δ 90.00 and carbonyl carbon of sydnone ring near δ 161.00. The other proton shift values of (**7a-j**) are indicating in experimental section. The antibacterial activity of all the synthesized compounds is shown in Table 1. Compound **7e** is found most active against *S. pneumoniae* and *P. aeruginosa*. Phenyl substitution at 4th position of phenyl ring increase activity against these two micro organism species while **7a** bearing methyl group at fourth position of piperazine ring shows highest activity against *S. aureus*. Compound **7i** bearing methyl group at second position of piperazine ring shows highest activity against *E. coli* while compound **7e** found most active against *P. Aeruginosa*. The yield of the compounds **7a-j** varies from 55-65 %, depending upon nature of amine component used.



Scheme 1: Synthesis of 3,3'-(methylenedi-1,4-phenylene)bis[4-{(4-substituted-amino) sulfonyl} sydnone (**7a-j**) from 4,4'-diaminodiphenyl methane. R = 7a: N-methyl piperazine; 7b: morpholine; 7c: diethylamine; 7d: diphenylamine; 7e: N-phenyl piperazine; 7f: imidazole; 7g: N-ethyl piperazine; 7h: *N*-(2,6-dichlorophenyl)piperazine; 7i: 2-methyl piperazine; 7j: piperazine.

Conclusion

The new sulfonamide derivatives of sydnone were synthesized and evaluated their antibacterial activity. The activity varies with the different substituent on sulfonamide linkage. It is noted that the potential of the compounds increase, minimum inhibitory concentration decreases. Most of the compounds found to possess antibacterial activity.

Table 1. Antibacterial activity of 3,3'-(Methylenedi-1,4-phenylene)bis(4-substituted-amino sulfonyl)sydnone (**7a-j**).

Compd. No.	**Gram Positive Organism**				**Gram Negative Organism**			
	S.pneumoniae		***S.aureus***		***E.Coli***		***P. aeruginosa***	
	IZ	**MIC**	**IZ**	**MIC**	**IZ**	**MIC**	**IZ**	**MIC**
7a	14	64	18	32	13	64	10	-
7b	12	128	13	128	16	32	12	128
7c	10	-	14	64	14	32	13	64
7d	14	32	16	32	18	16	12	128
7e	17	32	15	32	16	32	13	32
7f	14	32	15	32	16	16	10	-
7g	10	-	13	64	14	64	10	-
7h	12	64	10	-	15	32	13	64
7i	13	64	14	64	17	32	12	128
7j	13	64	10	-	14	64	13	64
Streptomycin	40	0.25	40	0.125	28	1.0	34	0.5
Penicillin-G	35	0.25	45	0.125	30	0.5	38	0.25

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Synthesis, characterization and biological activity of some novel benzimidazole derivatives

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***ABSTRACT**: The reaction of o-phenylenediamine with anthranillic acid yield compound 2-(1H-benzo[d]imidazol-2-yl)aniline (AOP). The compound AOP was condensed with aromatic acid chlorides in the presence of pyridine to get compound N-(2-(1H-benzo[d]imidazol-2-yl)phenyl)benzamide (AB). Further it to then treated with PCl_5 to get an intermediate compound then reacted with NaN_3 to yield compound 2-(2-(5-phenyl-1H-tetrazol-1-yl)phenyl)-1H-benzo[d]imidazole (ABC). The compounds were synthesized in good yields and their structures were confirmed by IR, ^{1}H-NMR, ^{13}C-NMR spectral data and elemental analysis. Antimicrobial activity against bacteria and fungi was studied. The results of preliminary biological tests showed that of these compounds possess good biological activities.*

***Keywords**: benzimidazole, anthranillic acid, o-phenylenediamine, PCl_5, NaN_3, biological activity*

Benzimidazole derivatives are very useful intermediates or subunits of the development of pharmaceutical or biological interest [1]. Benzimidazole derivatives are an important class of bioactive molecules in the field of drugs and pharmaceuticals [2]. Benzimidazole derivatives have found the application in diverse therapeutic areas including antiulcer, antihypertensive, antiviral, antifungal, anticancer, anti-histaminic [3], antitubercular [4], antiallergic [5, 6], antioxidant [7, 8], antimicrobial [9-11] and *in vitro* anti-HIV-1 [12] activities etc. A compound containing benzimidazole and benzene rings have been used extensively for pharmaceutical purpose since 1960. 1-H-Benzimidazole rings, which exhibit remarkable basic characteristics due to their nitrogen content,

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comprise the active substances for several drugs. A number of biological activities have been attributed to these compounds [13].

We elicited a new method for the synthesis of benzimidazoles with aromatic acid chloride. In continuation of our work on these different aromatic acid chlorides, we wish to report in the paper synthesis of 2-substituted benzimidazoles by the reaction between aromatic acid chlorides.

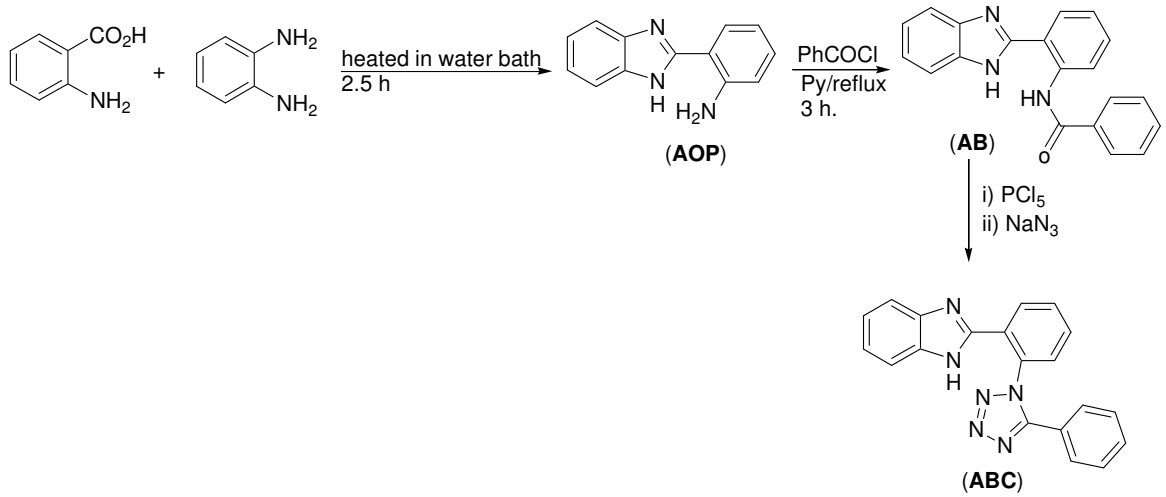
All the melting points were taken in open capillaries and are uncorrected. IR spectra were recorded in KBr on Shimadzu spectrometer, ^{1}H NMR and ^{13}C NMR in DMSO-d_6 on a Bruker AC-400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C), using TMS as an internal standard.

N-(2-(1H-benzo[d]imidazol-2-yl) phenyl)benzamide(AB): A mixture of compound (0.001 mol) of AOP and equivalent amount of benzoyl chloride (0.001 mol) was refluxed with pyridine (40 mL) for 3 hours. The reaction mixture was cooled, treated with cold ice and neutralized with conc. HCl. The separating solid was filtered and washed with ice cold water. The product was recrystalized from ethanol. Compound AB mp 187 ^{0}C (Found C, 76.64; H, 4.7; N, 13.39; O, 5.09% $C_{20}H_{15}N_3O$) IR: 3269 (N-H stretching), 3056 (aromatic C-H stretching), 1654 (C=O stretching), 1599 (C=N stretching), 1309 (C-N stretching), ^{1}H NMR: δ 7.26-7.85 (13H, m, Ar-H), 8.1 (1H, s, CO-NH), 11.9 (1H, s, imidazole ring NH), ^{13}C NMR: δ 115-128 (18C, Ar-C), 169 (1C, C=O), 150 (1C, C=N).

2-(2-(5-phenyl-1H-tetrazol-1-yl)phenyl)-1H-benzo[d]imidazole (ABC): A known amount of compound AB 0.01 mol (0.313 g) was taken in a beaker and added a known amount of PCl_5 0.01 mol (0.208 g) was heated at 100 ^{0}C until the evaluation of HCl fumes ceased. The reaction mixtures contain some unreacted $POCl_3$ this was removed by distillation under reduced pressure. The resulting was treated with ice cold solution of known weight of NaN_3 0.02 mol (0.130 g), a known volume of acetone 40 mL, known volume of sodium acetate was added. The reaction mixture was stirred over night. The acetone was removed by distillation under reduced pressure. The resulting mixture was extracted with $CHCl_3$ then the organic layer was separated and evaporated we got product. The product filtered and washed with ice cold water. The product was recrystalized from benzene and pet-ether mixture. Compound ABC mp 236 ^{0}C (Found C, 70.97; H, 4.14; N, 24.80; $C_{20}H_{14}N_6$) IR: 3270 (N-H stretching), 3056 (aromatic C-H stretching), 1655 (C=N stretching), 1313 (C-N stretching), ^{1}H NMR: δ 7.0-7.94 (13H, m, Ar-H), 11.8 (1H, s, imidazole ring NH), ^{13}C NMR: δ 114-131 (18C, Ar-C), 159 (1C, C=N in tetrazole ring), 150 (1C, C=N).

The reactions were carried out at room temperature, using aromatic acid chloride in the presence of pyridine, accordingly with Scheme 1.

Scheme 1



The antimicrobial activity for the given samples was carried out by Disc Diffusion Technique (Indian Pharmacopoeia 1996, Vol II A-105). The test microorganisms of Gram positive *Staphylococcus aureus* and Gram negative *Escherichia coli* and fungus *Candida albicans, Aspergillus Niger* were obtained from National Chemical Laboratory (NCL), India, and maintained by periodical sub culturing on Nutrient agar and Sabourad dextrose medium both bacteria and fungus respectively. The effect produced by the sample was compared with the effect produced by the positive control (Reference standard Ciprofloxacin 5 µg/disc for bacteria and Fluconazole 10 µg/disc for fungi). The result indicated that compounds were more active against all four organisms with reference to standard. The results are shown in the Table 1.

Table 1. Antimicrobial screening results of the compounds.

S. No.	Microorganism	Diameter zone of inhibition in mm		
		AB	**ABC**	**Std.**
1	*Staphylococcus aureus* (NCIM 2079)	25	32	40
2	*Escherichia coli* (NCIM 2065)	30	36	40
3	*Candida albicans* (NICM 3102)	15	17	20
4	*Aspergillus Niger* (NICM 105)	14	16	20

In conclusion, we have described a simple and efficient method for the synthesis 2-substituted benzimidazoles. The easy work up procedure, non-toxic cost efficiency providing this reactions scheme, excellent yields makes this method a valuable contribution to the existing methodologies.

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