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VIEWS

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Effect of different Properties on Self Assembly and Stability of Niosomes and Proniosomes: An Overview

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ABSTRACT

Vesicular drug delivery systems, have been studied since a lot of time, of which main importance was given to liposomes. Due to their unstability on storage, a lot of alternative materials were searched, of which niosomes are the primary ones, having a significant stability. These vesicles were formed from non ionic surfactants and were first reported in cosmetic industry. In this article a brief review is given on the different parameters affecting self assembly of niosomes and their stability. Also, the self assembly of surfactants into niosomes is governed not only by the nature of the surfactant but by the presence of membrane additives, the nature of the drug encapsulated and the actual method of preparation. Along with these, the different methods for preparation of niosmes and drug load optimization are briefly discussed. Further novel methods of presenting niosomes in the form of proniosomes, to avoid the step of freeze drying and have improved stability are mentioned. Finally the advantages of proniosomes and the applications of niosomal drug delivery system were enlisted.

KEY WORDS: Niosomes, Proniosomes, Cholesterol, Dicetylphosphate.

Non-ionic surfactant based vesicles (niosomes) are formed from the self-assembly of non-ionic amphiphiles in aqueous media resulting in closed bilayer structures (Fig. 1).

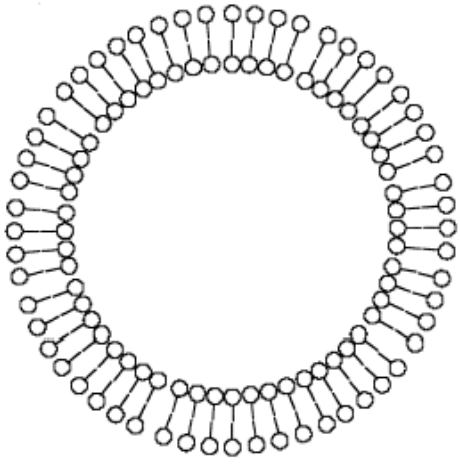

Fig. 1. Schematic representation of a niosome, O = hydrophilic head group, –– = hydrophobic tail

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The assembly into closed bilayers is rarely spontaneous [1] and usually involves some input of energy such as physical agitation or heat. The result is an assembly in which the hydrophobic parts of the molecule are shielded from the aqueous solvent and the hydrophilic head groups enjoy maximum contact with same.The low cost, greater stability and resultant ease of storage of non-ionic surfactants [2] has lead to the exploitation of these compounds as alternatives to phospholipids. Niosomes were first reported in the seventies as a feature of the cosmetic industry [3] but have since been studied as drug targeting agents.

The ultimate identity of any niosomal system and hence its properties are determined by the factors listed in Fig 2. It is thus obvious that all these variables must be carefully controlled in the design of a niosomal drug delivery system.

1. Factors governing the self assembly of non-ionic surfactants into niosomes

1.1. *Non-ionic surfactant structure*

Theoretically niosome formation requires the presence of a particular class of amphiphile and aqueous solvent. In certain cases cholesterol is required in the

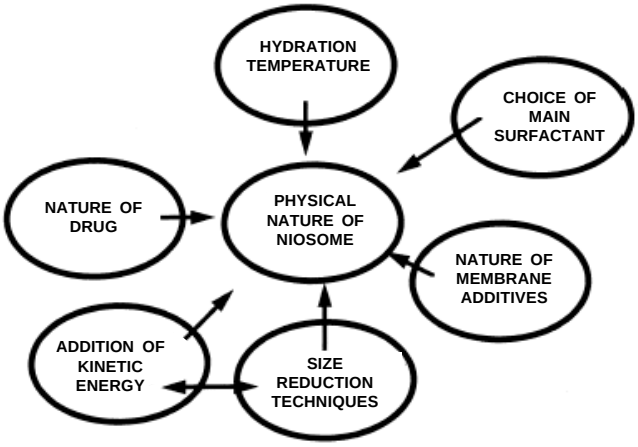

Fig. 2. Factors influencing niosome physical chemistry

formulation to increase the rigidity of vesicle and stop leakage. vesicle aggregation for example may be prevented by the inclusion of molecules that stabilise the system against the formation of aggregates by repulsive steric or electrostatic effects. An example of steric stabilization is the inclusion of Solulan C24 (a cholesteryl poly-24-oxyethylene ether) in doxorubicin (DOX) sorbitan monostearate (Span 60) niosome formulations [4]. Another example of electrostatic stabilisation is the inclusion of dicetyl phosphate in 5(6)-carboxyfluorescein (CF) loaded Span 60 based niosomes [6].

While the number of hydrophobic permutations is at present limited, there have been a wide variety of hydrophilic head groups in vesicle forming surfactants and it is in this area of vesicle forming surfactant design that considerable scope for new formulations still exist. The two portions of the molecule may be linked via ether, amide or ester bonds .

It has been observed that a parameter like the hydrophilic lipophilic balance (HLB) is a good indicator of the vesicle forming ability of any surfactant. With the sorbitan monostearate (Span) surfactants, a HLB number of between 4 and 8 was found to be compatible with vesicle formation [6]. The practical methods of HLB number determination have been reported [7]. These studies may be useful in the evaluation of new classes of compounds for their vesicle forming ability. The water soluble detergent polysorbate 20, also forms niosomes in the presence of cholesterol. This is despite the fact that the HLB number of this compound is 16.7 and it appears on first inspection to be too hydrophilic to form a bilayer membrane. However with an optimum level of cholesterol, it seems that niosomes are indeed formed from polysorbate 20 [8].

1.2. *Membrane addities*

Genaerally, there is a tendency of aggregation for vesicular drug delivery systems like liposomes and niosomes. Hence different membrane additives like Dicetyl phosphate and stearylamine are added to induce surface charge, thereby vesicular aggregation can be prevented. Here, Dicetyl phosphate induces negative charge, whereas stearylamine induces positive charge on the membrane [9].

1.3. *Nature of the encapsulated drug*

Another factor often overlooked is the influence of an amphiphilic drug on vesicle formation. While sorbitan monostearate (Span 60) niosomes containing dicetyl phosphate formed homogenous dispersions when encapsulating CF, this system formed an aggregated dispersion when encapsulation of the amphipathic drug DOX was attempted. A steric stabiliser Solulan C24 (poly-24-oxyethylene cholesteryl ether) must be added to the formulation to ensure a homogenous formulation devoid of aggregates [10]. DOX has been shown to alter the electrophoretic mobility of hexadecyl diglycerol ether (C16G2) niosomes in a pH dependent manner [11], an indication that the amphipathic drug is incorporated in the vesicle membrane.

1.4. *Surfactant and lipid levels :*

The level of surfactant:lipid used to make niosomal dispersions is generally 10–30 mM (1–2.5% w:w) [12]. Altering the surfactant:water ratio during the hydration step may affect the system's microstructure [13] and hence the system's properties. However increasing the surfactant:lipid level also increases the total amount of drug encapsulated, as discussed below, although highly viscous systems result, if the level of surfactant:lipid is too high.

1.5. *Temperature of hydration*

The hydrating temperatures used to make niosomes should usually be above the gel to liquid phase transition temperature of the system. [11].

2. Niosome preparation

The formation of vesicular assemblies requires the input of some form of energy and all the experimental methods surveyed consist of the hydration of a mixture of the surfactant:lipid at elevated temperature followed by optional size reduction to obtain a colloidal dispersion. This is followed by the separation of the unentrapped drug from the entrapped drug by either centrifugation, gel filtration or dialysis. Only one method (Novasome®) could be found in the literature on the preparation of niosomes on an industrial scale [14]. This involves the injection of the melted surfactants:lipids into a large volume of well-agitated heated aqueous solutions. Although a method involving the addition of an aqueous solution to a solid mixture of lipids and surfactants is said to be suitable for the handling of 'large quantities—kilograms' of dispersions.

2.1. *Hydration techniques*

The more commonly used laboratory methods of niosome preparation and drug loading identified in the literature are listed below.

1. The injection of an organic solution of surfactants:lipids in an aqueous solution of the drug to be encapsulated which is heated above the boiling point of the organic solvent (ether injection) [15].
2. The formation of a surfactant:lipid film by the evaporation of an organic solution of surfactants:lipids. This film is then hydrated with a solution of the drug (hand shaking). This method was previously described by Bangham and others [16] for the preparation of liposomes.
3. The formation of an oil in water (o:w) emulsion from an organic solution of surfactants:lipids and an aqueous solution of the drug. The organic solvent is then evaporated to leave niosomes dispersed in the aqueous phase. In some cases, a gel results which must be further hydrated to yield niosomes. (reverse phase evaporation) [17].
4. The injection of melted lipids:surfactants into a highly agitated heated aqueous phase in which presumably the drug is dissolved or the addition of a warmed aqueous phase dissolving the drug to a mixture of melted lipids and hydrophobic drug [18].
5. The addition of the warmed aqueous phase to a mixture of the solid lipids:surfactants [19].

2.2. *The reduction of niosome size*

Niosomes prepared as described above are usually in the micron size range although some of the methods produce niosomes in the sub-micron (:300 nm) size range. Often a size reduction step must be incorporated into the niosome production procedure, subsequent to the initial hydration step as vesicle size has an important bearing on vesicle biodistribution. For example sub-200 nm phospholipids vesicles have been shown to avoid splenic but not liver uptake. A reduction in vesicle size may be achieved by a number of methods.

1. Probe sonication [20] which yields C16G3 niosomes in the 100–140 nm size range.
2. Extrusion through 100 nm Nucleopore filters [21] which yields sodium stibogluconate C16G3 niosomes in the 140 nm size range.
3. In some instances the combination of sonication and filtration (220 nm Millipore® filter) has been used to achieve DOX loaded Span 60 niosomes in the 200 nm size range .
4. The achievement of sub-50 nm sizes is possiblen by the use of a microfluidizer.
5. High-pressure homogenisation also yields vesicles of below 100 nm in diameter although drug loading is ultimately sacrificed to achieve this small size.

2.3. *Drug loading optimization*

2.3.1. *Units for the reporting of drug load*

As expected drug loading is a crucial factor in the formulation of niosome delivery systems. However before a discussion on drug loading can begin, it is important to emphasise that due care and attention must be paid to the units used to quote drug-loading values. For example, drug loading values are often quoted as the % drug encapsulated. However for these values to have any meaning the initial drug, surfactant:lipid ratio must be stated. A simple study in which the amount of DOX encapsulated was measured as a function of the initial level of surfactant:lipid, showed that this initial surfactant:lipid ratio determines ultimately the % encapsulation. It was found that although the % encapsulation values steadily increased the final ratio of drug to surfactant:lipid decreased steadily. In a similar study a surfactant:lipid concentration ranging from 50–1000 mM showed no change in the final molar ratio of CF to surfactants:lipids although the '% encapsulation' increased steadily [5].

Clearly encapsulation efficiency once given in % encapsulation must be qualified with details on the initial ratio of drug to surfactant lipid. In our opinion the most useful value to any formulator will be the ratio of drug to surfactant in the final formulation in (g g_1) or (mol mol_1). This gives adequate information on the level of excipient that must be administered at each dose level.

2.3.2. *Methods of drug load enhancement*

Various techniques may be used to optimize drug load and this is especially important in industrial settings where there is limited scope for the chemical modification of excipients due to regulatory concerns. One such method is the dehydration – rehydration vesicle (DRV) technique first described by Kirby and Gregoriadis [22] which was found to increase the encapsulation efficiency of PK1 in C16G2 niosomes from 3.3 to 64.4%. Unfortunately niosome size was also doubled, increasing from 151 to 380 nm. A final PK1 to surfactant ratio of 0.3 was achieved with these DRV formulations. It was noted that the ease of rehydration of these freeze-dried dispersions was directly proportional to the phase transition temperature of the non-ionic surfactant. Other methods used to maximise drug loading include the use of pH gradients [23]. In this method a pH differential exists across the niosome membrane with a lower pH inside the niosome. The amine drug is then added external to the niosome and crosses the membrane barrier in the unionised state. Once inside the niosome the drug becomes protonated and is unable to leave the niosome. The acid pH within the niosome interior thus acts as an intra-vesicular trap. This method has been employed in the

formulation of vincristine sulphate niosomes [24] using citrate buffer (pH 4.0) followed by the addition of vincristine sulphate and the upward adjustment of the pH to 7.1. Once the pH has been adjusted upwards, the formulation is heated above the phase transition temperature (60°C) of the membrane in order to increase vesicle permeability.

2.4. *Separation of entrapped material*

The hydration of surfactant:lipid mixtures rarely leads to the entire drug being encapsulated, regardless of the drug loading optimisation steps taken. It is thus often a requirement that unencapsulated drug be removed by various means. Although it may be argued that the use of systems in which half of the drug is encapsulated and half is external to the niosome may eventually yield systems with a beneficial biphasic biodistribution profile. This drug delivery system would give an initial burst to initiate therapy followed by a sustained maintenance dose. This is demonstrated by the improved activity against *Leishmania dono*6*ani* seen with alkyl polyglycerol or alkyl polyoxyethylene based sodium stibogluconate niosomes when unentrapped drug was not removed when compared with niosomes in which the unentrapped drug had been removed. These former formulations were also superior to the use of the free drug.

The methods that have been used for the removal of unentrapped material include:

1. Exhaustive dialysis [25].
2. Separation by gel filtration (Sephadex G50) [10].
3. Centrifugation (7000_*g* for 30 min) for DOX C16G3 niosomes prepared by hand-shaking and ether injection methods [26].
4. Ultracentrifugation (150000_*g* for 1.5 h) for PK1 niosomes [27].

3. Niosome stability

It would be unwise not to include a separate discussion of niosome stability in this review although it must be borne in mind that all the material presented above relate to or have a direct influence on the stability of niosomal dispersions. A stable niosome dispersion must exhibit a constant particle size and a constant level of entrapped drug. There must be no precipitation of the membrane components, which are to a large extent not insoluble in aqueous media. Ideally these systems should be stored dry for reconstitution by nursing staff or by the patient and when rehydrated should exhibit dispersion characteristics that are similar to the original dispersion.

3.1. *Influence of the surfactant:lipid nature*

The choice of membrane surfactant determines the nature of the membrane and ultimately affects the stability of the system. The leakiness of CF loaded Span surfactant niosomes was found to follow the trend Span 80<Span 20<Span 40< Span 60 [5] and was determined by the degree of membrane fluidity. The incorporation of cholesterol into these niosomal systems also decreases the leakiness of the membrane.

3.2. *Influence of the encapsulated drug*

The encapsulated drug could also be the major determinant of the fate of any niosomal system. 75% of the drug polymer conjugate (PK1) remained encapsulated within the vesicles 28 days after storage as the vesicle suspension at 4 and at 25°C. Vesicle size was also found to remain unchanged [28].The encapsulation of a polymer obviously leads to a more stable system as the membrane is suffi-ciently impermeable to this macromolecule. The physical nature of the encapsulated material also affects stability. DOX loading into vesicles using an ammonium sulphate gradient is said to lead to the formation of a gel within the vesicles. Niosomes loaded using this technique were also less leaky.

3.3. *Temperature of storage*

The temperature of storage of these dispersions must be controlled as a change in the temperature of the system often leads to a change in the fundamental nature of the system or an increase in the release of an encapsulated solute [8] a property which may be exploited to construct a thermoresponsive system.

3.4. *Detergents*

High concentrations of detergents (soluble surfactants) are incompatible with niosomal systems and cause eventual solubilisation of the vesicles to form mixed micelles and a host of intermediate aggregates. This solubilisation has been studied for a few formulations and the destruction of C16G2 niosomes by octyl glucoside appears to proceed via the build up of a critical localised concentration of octyl glucoside molecules within the niosome membrane before micellisation can occur. The solubilisation C16G2 niosomes by Solulan C24 has been shown to proceed via the formation of discomes which are then converted into mixed micelles [29].

3.5. *Stability enhancement*

Methods to enhance the stability of these niosomes are also found in the literature. Decreasing the air water interface may prevent the crystallization of these self assembled surfactant monomers and it may be possible to stabilize niosomes by a variety of methods such as the addition of polymerised surfactants to the formulation, the use of membrane spanning lipids and the interfacial polymerisation of surfactant monomers in situ. The inclusion of a charged molecule in the bilayer shifts the electrophoretic mobility making it positive with the inclusion of stearylamine and negative with the inclusion

of DCP and also prevents niosome aggregation. In addition, as mentioned above, the entrapment of hydrophobic drugs or macromolecular prodrugs also increases the stability of these dispersions [30].

4. Proniosomes

The traditional method for producing niosomes or liposomes involves drying the lipid to a thin film from organic solvent, and then hydrating this film with the aqueous solvent of choice [31]. The resulting multilamellar vesicles can be further processed by sonication, extrusion, or other treatments to optimize drug entrapment . Other methods, such as injection of lipids in water-miscible or water-immiscible solvents into an aqueous solution, detergent dialysis, or reverse-phase evaporation are complicated by the need to remove certain components following liposome formation . All of these methods are time consuming, and many involve specialized equipment. The thin film approach allows only for a predetermined lot size so material is often wasted if smaller quantities are required for a particular application or dose.

Proniosomes [32] circumvent all of these complications. These are dry formulations of surfactant-coated carrier, which can be measured out as needed and rehydrated by brief agitation in hot water. Proniosomes (and proliposomes) are normally made by spraying surfactant in organic solvent onto sorbitol powder and then evaporating the solvent. Because the sorbitol carrier is soluble in the organic solvent, it is necessary to repeat the process until the desired surfactant loading has been achieved. The surfactant coating on the carrier is very thin and hydration of this coating allows multilamellar vesicles to form as the carrier dissolves. The resulting niosomes are very similar to those produced by conventional methods and the size distribution is more uniform. It was suggested that this formulation could provide a suitable method for formulating hydrophobic drugs in a lipid suspension without concerns over instability of the suspension or susceptibility of the active ingredient to hydrolysis.

4.1. Methods of preparation :

1) Spraying method :

In this method carriers like sorbitol, mannitol are used, on which organic solution of lipids and surfactants are coated slowly. Here the application of solution should be slow , because the carrier is soluble in the organic solvent [33].

2) Slurry method :

Here an insoluble carrier like maltodextrin is used, on which the whole of the organic solution is poured and rotated in a rotary vaccum evaporator to get freely flowing proniosomes [34].

3) Gel method :

In this method a gel is formed, instead of powder. Generally this method is used to prepare transdermal formulations. Generally lecithin is used as a surfactant in this method [35].

4.2. Advantages:

- Stability is very high when compared to liposomes and niosmes.
- Ease of manufacture and commercial scale up.
- No need of freeze drying as compared to niosomes.
- Highly economic, due to ease of preparation.

5. Biomedical Applications

Although pharmaceutical niosome formulations have yet to be commercially exploited, a number of studies have demonstrated the potential of niosomes in drug delivery. Lot of study has been taken place to evaluate the use of niosomes and proniosomes.

1) Anti infective agents :

Indeed one of the earliest diseases for which niosomal formulations proved particularly beneficial was from the antiparasitic class, specifically in the treatment of experimental leishmaniasis. The intravenous administration of sodium stibogluconate C16G3 niosomes or dipalmitoyl phosphatidylcholine (DPPC) liposomes both containing 30 and 20% cholesterol, respectively, resulted in higher liver levels of antimony when compared with the administration of the drug in solution. These niosomes were prepared by the ether injection method and thus are presumed to be in the 300nm–1 mm size range [25].

2) Anti cancer drugs :

When methotrexate 100 nm C16G3 niosomes containing either 47.5 or 30% cholesterol were administered intravenously or orally higher levels of the drug were found in the liver—more so for the formulations administered by the intravenous route—with serum levels higher than when the drug was administered in solution. A 23-fold increase in the area under the plasma level time curve was observed when Span 60 4.5 mm methotrexate niosomes were administered by the intravenous route to tumour bearing mice a fact attributed to the large size of these niosomes [36].

3) Anti inflammatory agents :

Diclofenac niosomes reportedly prepared from polysorbate 60, cholesterol and DCP (22:73:5) and 3 mm in size were found to reduce the inflammation in rats with carageenan induced paw oedema on intraperitoneal administration to a greater extent than the free drug. This increase in activity is a direct result of an observed increase in the area under the plasma time curve [37].

4) Diagnostic imaging :

Apart from the use of niosomes as various drug carriers one report in the literature details the evaluation of these systems as diagnostic agents.C16G3 and C16C12G7 niosomes containing cholesterol and stearylamine encapsulating the radioopaque agent iopromide were found to concentrate in the kidneys on intravenous administration. This kidney targeting was attributed to the presence of the positive charge on the niosome surface although no neutral control niosomes were used in this study [38].

5) Niosomes as vaccine adjuvants :

A number of surfactants have documented immunostimulatory properties and have been used in emulsion vaccine adjuvants. The adjuvanticity of niosomes prepared from 1-mono-palmitoyl glycerol, cholesterol, dicetyl phosphate—5:4:1 has been demonstrated in mice, on subcutaneous administration of bovine serum albumin ovalbumin or a synthetic peptide containing a known T-cell epitope. The encapsulation of the antigen was determined to be crucial to the adjuvanticity. The same niosome system has also been shown to act as a vaccine adjuvant when administered intraperitoneally to severe combined immunodeficiency mice reconstituted with peripheral blood lymphocytes (PBL-SCID mice) [39].

6) Transdermal drug delivery :

Although the emergence of niosomes into the pharmaceutical arena was the result of activity in the cosmetic industry, it was only fairly recently that the transdermal delivery of drugs with niosomes was seriously considered. The enhanced delivery through the stratum corneum of niosome encapsulated drugs has been observed and it therefore remains to elucidate the mechanism of this delivery, especially as the stratum corneum is considered to be a particularly impermeable barrier. Small (100 nm) vesicular structures have been observed between the first and second layer of human corneocytes 48 h after incubation with niosomes prepared from 'dodecyl alcohol polyoxyethylene ether' and cholesterol. Penetration by niosomes of this upper layer appears plausible as these layers are only loosely packed [40].

7) Opthalmic drug delivery :

A single study reports on the biological evaluation of a niosomal drug delivery system for ophthalmic delivery. Cyclopentolate was encapsulated within niosomes prepared from polysorbate 20 and cholesterol and found to penetrate the cornea in a pH dependant manner within these niosomes. Permeation of cyclopentolate increased at pH 5.5 but decreased at pH 7.4. Contrary to these findings, in vivo there was increased mydriatic response with the niosomal formulation irrespective of the pH of the formulation. It is concluded that the increased absorption of cyclopentolate may be due to the altered permeability characteristics of the conjuctival and scleral membranes [41].

Conclusion

A number of hydrophilic units may be used to synthesise vesicle forming non-ionic surfactants. While the correlation of head group chemistry with vesicle physical chemistry and biology remains to be systematically carried out, it is evident that a rich array of vesicular structures may be produced from a variety of as yet unsynthesised compounds. Niosomes have been proven to be useful in the delivery of anti-infective agents, anti-cancer agents anti-inflammatory agents and fairly recently as vaccine adjuvants. These systems have been proven to target certain areas of the mammalian anatomy and may be exploited as diagnostic imaging agents. All this is supremely encouraging for further research in this arena of drug delivery.

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Biotransformation of the 1, 8-cineole by *Rhizopus arrizus*

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ABSTRACT

This paper reports biotransformation of 1, 8-cineole to its different hydroxyl derivatives using free cells of rhizopus species. Microorganisms were examined for their potential to hydroxylate the oxygenated monoterpene 1, 8-cineole.Using gas chromatography and thin-layer chromatography, screening experiments revealed that hydroxylation at position 2 was the most commonly observed microbial transformation reaction.Preparative-scale biotransformation with rhizopus cell suspensions resulted in the production of three different optically pure compounds, which were identified as 2-*endo*-hydroxy-1,8-cineole, 2-*exo*-hydroxy-1,8-cineole and 2-oxo-1,8-cineole based on nuclear magnetic resonance and mass spectral analyses. The culture preparation variables such as pH, temperature and incubation period for obtaining maximum cell growth and product concentration from *Rhizopus arrizus* were optimized. The optimized culture conditions for free cells of *R. arrizus* have been compared for product biotransformation. The various factors such as the optimum substrate concentration and the time of substrate addition at varying cell concentrations during the growth of fungal culture were also studied. Highest product concentration of 2.15 gl^{-1} was obtained with free cell catalyzed biotransformation at pH 6.0, 27ºC and 150 rpm after 96hrs using 1, 8-cineole initial concentration of 4.0 gl^{-1}.

KEY WORDS: Microorganism, Biotransformation, 1, 8-cineole, *Rhizopus arrizus*, Hydroxyl derivatives

Introduction

Monoterpenes are widely distributed in nature and find extensive applications in the flavor and fragrance industry. Their simple structures make them ideal targets for microbial biotransformations to yield several commercially important products [1]. 1, 8-cineole (1, 3, 3-trymethyl-2-oxabicyclo [2.2.2] octane), a monoterpene cyclic ether, is one of the main components in essential oils from *Eucalyptus globulus* and Eucalyptus *polybractea* which bears distinct odor characteristics [2]. The oxidized derivatives of 1,8-cineole represent a set of compounds of high potential as chiral synthon for organic chemistry. Besides, several oxygenated terpenes have shown wide utility in the scent industry as a consequence of their fragrances [3,4]. Therefore, hydroxylation of 1, 8 cineole would increase its market value [5]. Production of these derivatives implies the stereo specific introduction of molecular oxygen in not activated carbon atoms, which continues to be a challenge in organic synthesis [6]. The use of microorganism that carries out this type of reactions constitutes an interesting alternative. Microbial hydroxylations have advantages over classic organic synthesis procedures since they are carried out in soft conditions, they use biodegradable reagents and they are generally stereo selective resulting in the production of an optically pure synthon [7,8].

It would be interesting to generate hydroxy derivatives via bioconversion of 1, 8 cineole. The product generated via biotransformation is labeled as a 'natural' product and commands higher value in the market. The biotransformation of other monoterpenes *i.e.* (D)-citronellal to (D)-citronellol using *Pseudomonas aeruginosa* [9] and *Saccharomyces cerevisiae* [10,11] have been reported. However, information on the biotransformation of 1, 8 cineole remains limited. The optimization of process parameters remains a challenging task due to several limitations posed by monoterpenes such as toxicity and volatility, by-product formation, immiscibility and low yields of the product.

In the present work, the optimal conditions for *Rhizopus arrizus* growth and its application in biotransformation of 1, 3, 3-trymethyl-2-oxabicyclo[2.2.2] octane to hydroxy derivatives by free cells suspensions is reported. The suitable culture conditions such as pH, temperature and agitation were studied to maximize the product concentration.

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Materials and Methods

Chemicals

Potato dextrose agar, malt extract, peptone, yeast extract, were purchased from Hi-Media Laboratories Pvt. Ltd. Mumbai, India. The substrate 1, 8, cineole was purchased from Sigma Chemicals Co., USA. Other chemicals of analytical grade were obtained from standard sources. 0.1 M Acetate buffer (pH 6.0.) was prepared.

Microorganism and cultural conditions

Strain of *Rhizopus arrizus* (ATCC10260) was obtained from National Collection of Industrial Microorganisms, NCL, India and maintained on potato/dextrose/agar slants at 4ºC. For cultivation in liquid media, growth from slants was inoculated in 100 ml sterile inorganic mineral media in 250 ml Erlenmeyer flasks and incubated at 27ºC, pH 6.0,150 rpm for 6 days.

Growth

The best media for growth was selected on the basis of dry cell weights calculated as below. *Rhizopus arrizus* cell suspension was prepared by suspending growth from slants. The cell concentration of suspension was adjusted to optical density 0.6 at 600nm and 0.2ml of this inoculum was used for subculturing 100 ml media (pH 6.0) in 250 ml of all the experimental Erlenmeyer flasks. And the flasks were incubated at 27ºC and 150 rpm.

Cell growth in all the media was determined by estimating the dry cell weight after every 9 hrs interval. This was done by separation of cells from the broth by centrifugation at 25ºC, 800 rpm for 30 min. The cells were added to pre-weighed aluminium foil and dried at 100ºC for 24 hrs. The difference in the initial and the final weights gave the dry cell weights. On obtaining the dry cell weight values, the growth curves [Dry wt. (gl^{-1}) vs. Time (hrs)] were plotted. The optimum pH for growth was determined by growing *R. arrizus* culture at 27ºC in modified PDB at different pH values (4 - 9). Suitable temperature for growth was determined by growing *R. arrizus* cells at pH 6.0 and different temperatures (20ºC - 30ºC). All the growth determination experiments were performed in triplicates.

Biotransformation

Biotransformations of 1, 8-cineole was carried out by fermentation under different conditions. Optimized methodology for biotransformation: *R. arrizus* strain was plated in liquid broth and incubated for 4 days (OD value equal to 0.8) at 27ºC. From this 0.2 ml culture was used to inoculate 100 mL liquid broth and the culture was incubated in an orbital shaker at 27ºC, 150 rpm for 3days were the cells reach early exponential phase. To this growing culture substrate was added and fermentation was continued under similar conditions [12,13].

Optimum conditions for biotransformation

250 ml Erlenmeyer flasks were used for obtaining *R. arrizus* growth in 100 ml media for studying optimum conditions necessary for biotransformation. The determination of suitable growth phase in modified inorganic mineral media was done and cells at early log phase were employed for biotransformation. This was done by harvesting cells at different phases of growth and measeuring their O.D values. The optimum values of pH and temperature were estimated in the range of pH 4 - 9 and 20ºC - 30ºC. The time required for maximum product formation was determined by analyzing the product after every 8hrs interval till 96hrs. FAn agitation speed of 150 rpm was employed. Optimum solvent/emulsifier type and concentration to be employed was standardized [14,15].

Extraction of metabolites

In order to produce sufficient amounts of metabolites for isolation and verification of structure. Cultures were grown in 200 ml of medium held in 500ml culture flasks, and a total of 4g of 1, 8-cineole substrate was used for 1000ml culture broth. Samples were taken and analyzed as described for screening experiments, and cultures were harvested between 48 and 96hrs after substrate addition. Harvested cultures were extracted with 3 500ml volumes of dichloromethane. The organic extracts were combined, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The reaction products were separated by repeated chromatography over silica gel and alumina. Products were separated on silica gel chromatographic column using hexane-ethyl acetate (17:3 vol/vol). Elution volume of 900ml yielded 630 mg of a mixture which was further purified on alumina column. Hexane-ether (1:4, vol/vol) solvent system was used for products isolation,. Total yield was 430mg from the solvent volume of 820 ml. The separated products were analyzed by HPLC, GC-MS and proton NMR for characterization [19, 20].

Identification of obtained products

Each compound was examined for purity by thin layer chromatography (TLC), gas chromatography mass spectral evaluation and proton nuclear magnetic resonance (NMR). Compounds used in this study included the following: 1, 8-cineole (compound1), 2-exo-hydroxy-1, 8, cineole (compound 2), 2-endo-hydroxy-1,8-cineole (compound 3) 2-oxo-1,8-cineole (compound 4)[16,17,18].

Chromatography:

TLC studies were performed on 0.25-mm-thick silica gel G (Merck) plates, to identify the metabolites. Solvent systems: hexane-ethyl acetate (17:3, vol/vol) is the elution solvents. Developed TLC plates after drying were sprayed with a solution of p-anisaldehyde-glacial acetic acid-concentrated sulfuric acid (0.5:60:0.5, vol/vol) and warmed in an oven at 50^0C for 5 minutes to develop colors.

Infra Red Spectroscopy

IR spectra were recorded from the NICOLET-AVATAR 330- FTIR. IR spectra obtained will be of help in identifying hydroxyl and keto group, which predominately differentiate the products from its substrate. And also spectra was particularly usefull in establishing the complete substrate utilization.

GCMS analysis.

The products were identified using a gas chromatograph coupled to a mass spectrometer (GC/MS) to determine the molecular weights of the products and ions formed by the fragmentation of their molecules. For the GC-MS analysis conditions were, unit: Thermo GC 2000 series, equipped with a DB-5 fused silica column (30m x 0.25mm i.d.; film thickness 0.25 µm) interfaced with a Saturn-3 Ion Trap Detector (ITD). Oven temperature: 70ºCfor 3 min with rise at the rate of 3ºC/min up to 250ºC kept isothermal for 2 min. The transfer line temperature was 280ºC. Helium was used as the carrier gas, flow rate was 1 mL/min and chemical ionization took place at 70eV. Split ratio 1:50; ionisation scan range 40-600 *m/z*.

Proton NMR

NMR spectra were recorded with Bruker WH-360 (360.134 MHz for proton NMR) NMR spectrometer. Spectra were all recorded in deuterochloroform solutions by using tetramethylsilane as an internal standard. Proton NMR spectral data obtained in $CDC1_3$ for various compounds, together with their assignments, have been reported in detail.

Results

Growth conditions

The suitable culture conditions necessary for obtaining maximum *R. arrizus* cell growth at shake flask conditions were determined. The increase in cell concentrations during growth of *R. arrizus* cells in modified inorganic mineral media was assessed using both dry cell weight, OD mesurement and plotted as growth curves (figure 1, 2). *R. arrizus* cells attained highest concentrations of 16.3 g l^{-1} modified mineral media. The *R. arrizus* cell growth rate values calculated in the exponential phases during culture was 0.086 h^{-1}. Determination of suitable pH values and temperatures for *R. arrizus* cell growth was done by culturing cells in the range of pH 4 -9 and 20ºC - 30ºC respectively. Higher cell concentrations of 16.2 gl^{-1} were obtained at pH 6.0. The cell concentrations gradually decreased at lower or higher pH values. Studies on different cultivation temperatures showed that the cell growth increased gradually from 20ºC till 27ºC, which was found to be most conducive for growth where optimum cell concentration of 16.1 gl^{-1} was obtained. Cells cultured at 30ºC showed lowered optimum cell concentrations (14.7gl^{-1}).

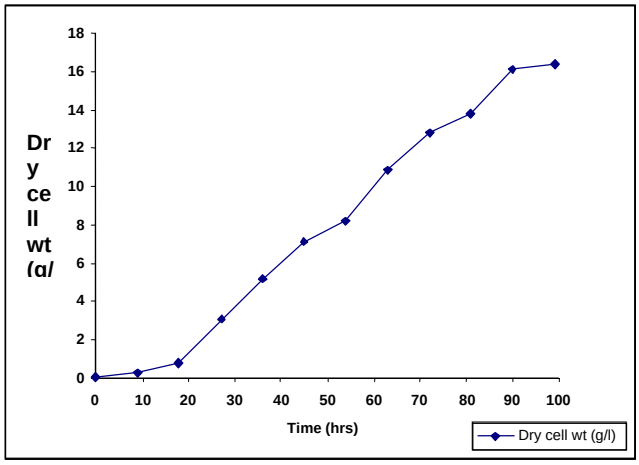

Fig.1. Growth measured as dry cell weight of R. arrizus at pH 6.0, 27 ⁰ C, 150 rpm for 96 hrs.

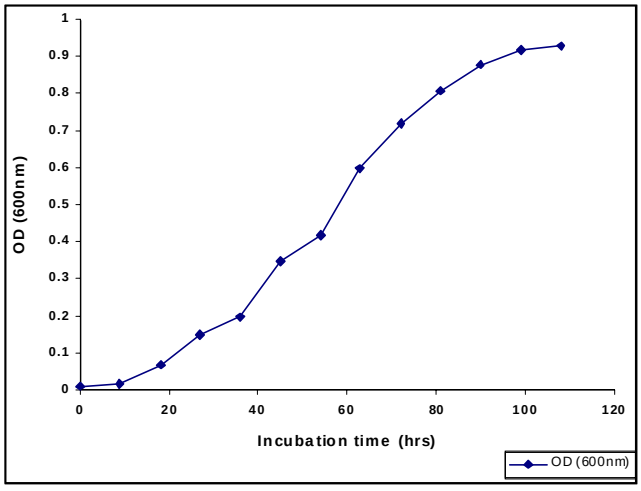

Fig. 2. Growth measured as OD (600nm) of R. arrizus at pH 6.0, 27 ⁰ C, 150 rpm for 96 hrs.

Culture conditions for biotransformation with free cells

The biotransformation of substrate cineole by free cells of *R. arrizus* was studied under different pH and temperature conditions. Biotransformation at pH values in the range of pH 4-9 showed a gradual increase in the product concentrations from pH 4 pH 6.0. No significant change in the product concentrations were seen at pH's above 6 at substrate concentration of 4.0 gl^{-1} (Figure 3.). No change in the pH value was seen during the course of biotransformation at the end of 8 hrs. Studies on the effect of temperature showed that the product concentration increased from 20ºC to a maximum of 2.1 ± 0.2 gl^{-1} at 27ºC and then decreased (Figure 4.).

For ascertaining the suitability of modified inorganic mineral media as growth and reaction media, biotransformation was carried out in different media combinations. Biotransformation in other media resulted in decreased product formation. Mineral media was found to be the suitable media composition for obtaining maximum

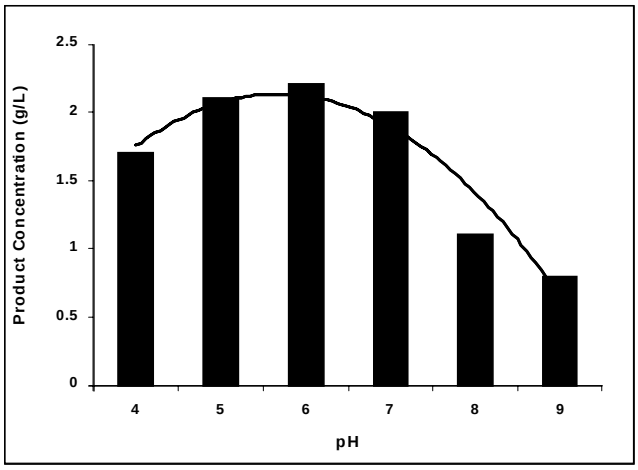

Fig. 3. Change in product concentrations at different pH (4-9) values during biotransformation by R.*arrizus* at 27^{0}C, 150rpm.

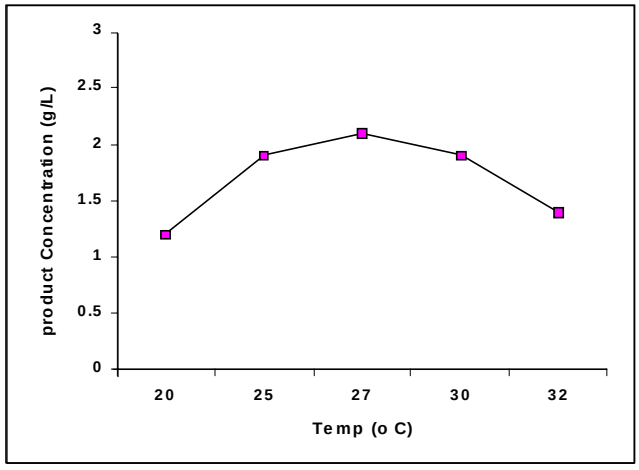

Fig. 4. Evaluation of the biotransformation product profile as a function of temperature between 20- 32 ^{0}C. 27^0 C temperature was found optimum.

product concentration. The determination of suitable culture age during growth of *R. arrizus* in PDB for maximum product formation was done by utilizing the cells in various stages of growth and employing them for biotransformation reaction. The results indicated the cell culture age of 40 hrs - 44 hrs to be suitable for optimum product formation. Here, the cells were at the early stage of the exponential phase and had attained good cell concentration. The maximum substrate concentration of 4.0 gl^{-1} could be optimally biotransformed to 2.15 $gl^{-1} \pm 0.1$ of total product. Marginal decrease in product concentration was observed on further incubation to 96hrs.

Identification of obtained products

Chromatographic properties of various 1, 8 cineole derivatives

Compound	Adsorbent	Color of the eluted compound	TLC Rf values
C1	Silica with gypsum	Purple	0.67
C2		pink	0.13
C3		pink	0.14
C4		Pinkish yellow	0.51

Spectral data

The hydroxylation of compound 1 to hydroxyl cineole compound 2,3 and 4 by biotransformation reaction was confirmed by spectral data. Results obtained from IR, GC-MS, and H NMR spectra reveal the formation of two hydroxy and one oxo derivative.

Infra Red spectroscopic spectra by KBr method showed the frequency maximum 3385 cm-1 (OH). High resolution EI-MS showed M^+ 170.1328 ($C_{10}H_{18}O_2$) with significant fragments of m/z 126 (M+ -CH2 =CHOH), 111 (M+-CH2=CHOH-CH3) and 43 (CH3C=O+). And 1 H NMR (ppm) signals at ϕ 3.72 (1H, ddd, HCOH) and ϕ 2.52 (1H, m, C3 –H endo) indicate that an OH group is attached to the C-2 of 1,8-cineole. 2-endo hydroxyl cineole IR by KBr method showed the frequency maximum 3480cm-1(OH), 1058 (alcoholic C-O) and m/z values M^+170, 153, 127, 112, 109 and 71 are the major fragments. And 1 H NMR (ppm) signals at ϕ1.11 (3H,s, CH_3 at C_7) and ϕ 1.99 (2H, m, exo-proton signal at 3.72 collapsed to d) indicate that an OH group is attached to the C-2 of 1,8-cineole. 2-oxocineole IR by KBr method showed the frequency maximum 1730 cm-1 (c=o), 1150 (c-0-c) cm-1. High resolution EI-MS showed M+ 168.11 ($C_{10}H_{16}O_2$) with significant fragments of m/z 140, 111, 83, 82, 71, 69, 67 and 43. 1 H NMR (ppm) signals at ϕ3.72 (1H, ddd, HCOH) and ϕ 1.15 (3H, s, CH3 at C-7), 1.24 (3H, S, CH3 at C-9) and 2.21 (1H,dd) indicate that an keto group is attached to the C-2 of 1,8-cineole.

Discussions

Microorganisms were examined for their abilities to hydroxylate the pleasant-smelling liquid monoterpene 1,8-cineole(C1).Thus, it is possible to envision that, as hydroxylation of 1,8-cineole occurred, the reaction was driven to completion. Analytical methods, including UV, TLC, IR, GC-MS, H NMR were established to permit the ready identification of various metabolites expected in microbial transformation experiments. TLC on silica gel plates was used in the general screening of microorganisms for their abilities to hydroxyl ate 1, 8-cineole. This system enabled the separation of all expected products. The identities of the metabolites were confirmed by conducting 1,8-cineole biotransformation reactions on a preparative scale with different microorganisms, reported species which appeared to perform reactions in good yield. The reaction products were isolated by solvent extraction and separated by column chromatography, and the isolated products were identified by H NMR and GC-MS.

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Preliminary Phytochemical and Antimicrobial Activity of *Croton sparsiflorus* Morong

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ABSTRACT

The different fractions of methanolic extracts of the whole plant of *Croton sparsiflorus* was subjected to preliminary phytochemical and *in-vitro* anti-microbial studies. The different fractions revealed the presence of steroids, alkaloids, flavonoids and saponins. The antimicrobial activity of the plant different fractions of methanolic extract was assayed by the agar plate disc diffusion and nutrient broth dilution techniques. Three gram negative, four gram positive bacterial and four fungal species were screened for the anti-microbial investigations. The fraction II of the methanolic extract inhibited the growth of all the test bacterial species whereas fraction III and fraction IV have shown weak antibacterial activity.

KEY WORDS: Antifungal, antimicrobial, *Croton sparsiflorus,* Phytochemical.

Introduction

Croton sparsiflorus Morong (Syn. *C. bonplandianum* Baill), Euphorbiaceae, is common weed throughout the plain of India. It is traditionally used for its wound healing activity. It is reported to be hypotensive and leaves are analgesic [1]. Earlier phytochemical work indictates that benzene soluble fraction of ethanol extracts of leaves and stem contains β-sitosterol, teraxerol [2], and neutral fraction contains vomifoliol [3]. The leaves of *C.sparsiflours* contain flavonoids Quercetin-3-rhamnoglucoside [4]. The ethanolic extract of the plant reported to contain proaporphine alkaloidal bases like crotosporin, N-methyl crotosporin, N, O,-dimethyl crotosporin [5]. Although phytochemical screening of this plant has been carried out, no extensive pharmacological activities have been reported on this plant. In the present study, different fractions of the methanolic extracts of whole plant have been investigated.

Materials and Methods

Plant material

The *Croton sparsiflorus* weed collected from Shimoga district of Karnataka state. The taxonomical identification of the plants were done by Dr. Manjunatha, Department Botany, SRNM College, Shimoga, and the voucher specimen was deposited at the Department of Pharmacognosy, National College of Pharmacy, Shimoga, for future reference.

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Preparation of extracts

The plant was washed with water and shade dried. The dried materials were powdered and passed through No.10 mesh sieve. The powdered plant material was subjected to extraction as per the standard scheme for preparation of plant extracts for biological screening [6]. The powdered plant materials were macerated with 80% methanol and the solution was concentrated under vacuum at 40° C. The residue was partitioned between chloroform and 10% citric acid solution. Chloroform layer was separated and evaporated in vacuum at 40° C. The residue was partitioned between 90% methanol and petroleum ether (1:1). The petroleum ether layer (fraction 1) and methanolic layer (fraction 2) were separated and concentrated under vacuum at 40° C. The aqueous citric acid layer was evaporated and concentrated. To the residue, ammonium hydroxide (P^H 9) was added and further extracted with chloroform. Chloroform layer (fraction 3) and aqueous layer (fraction 4) were separated and concentrated under vacuum at 40° C.

Preliminary phytochemical studies

All the fractions were subjected to preliminary phytochemical investigations for the presence of secondary

metabolites such as steroids, Triterpenoids, flavonoids, alkaloids, tannins, saponins, and resins utilizing standard methods of analysis [7].

Table 1:
Phytochemical screening of different fractions of methanolic extract of *Croton sparsiflorus*

Secondary metabolites	Frac–tion 1	Frac–tion 2	Frac–tion 3	Frac–tion 4
Sterols	+	–	–	–
Triterpenoids	+	–	–	–
Flavonoids	–	+	–	–
Alkaloids	–	–	+	–
Tannins	–	–	–	–
Saponins	–	–	–	+
Resins	–	–	–	–

+ Present; – Not detected.

Fungal and bacterial strains

Tests were performed on four fungi (*Candida albicans, Aspergillus niger, Aspergillus flavus* and *Rhizopus oligosporus)* and seven bacterial strains (*Escherichia coli, proteus vulgaris, Pseudomonos aeroginosa, Bacillus subtilis, Bacillus pumilus, Staphylococcus aureus* and *Streptococcus faecalis*) obtained from Department of Microbiology and Biochemistry, National college of Pharmacy, Shimoga, India.

Antibacterial screening

Working bacterial inocula suspensions were obtained from 18h stock culture on nutrient broth at 37^0 C. The inoculum size of each test strain was standardised at 5 x 10^5 cfu/ml, according to the National committee for clinical Laboratory standards [8, 9]. A 5ml volume of the bacterial suspension was evenly mixed with sterile nutrient agar medium and poured into the sterile Petri plates. After allowing the media to solidify at room temperature, wells of 6mm diameter were bored in the agar with sterile cork-borer. Each fraction was checked for antibacterial activity by introducing 40µl of a 50 mg/ml concentration into wells. The method was repeated in five plates. The plates were allowed to stand at room temperature for 1h for extract to diffuse into the agar media and then incubated at 37^0 C for 18h in BOD incubator (S.M Industries, New Delhi). Subsequently, diameter of zone of inhibition was measured. A broad spectrum antibiotic Ampicillin (40µg/ml) was used as the reference standard in each plate. Minimum inhibitory concentration (MIC) was determined by macro-broth dilution method[8]. The reconstituted extract was serially diluted two fold in nutrient broth medium. Five tubes of each dilution were inoculated with 5 x 10^5 cells (cfu) of the test bacterial strain and culture incubated in a water bath at 37^0 C for 18 h. MIC was taken as the lowest concentration of extract (highest dilution) showing no detectable growth.

Antifungal screening

5 ml of the fungal spore suspension was mixed evenly in SDA media and poured into the sterile petri plates and allowed to solidify at the room temperature. Wells of 6mm diameter were bored by using sterile cork-borer. All the fractions were tested for antifungal activity by introducing 40µl of a 50mg/ml concentration in to wells. The plates were incubated at 25^0 C for 3 days. A marketed sample of Nystatin (50IU/ml) was used as the reference standard. Subsequently zone of inhibition was measured to determine the antifungal activity of the extracts [10].

Table 2:
Antimicrobial activity of different fractions of *methanolic* extracts of *Croton sparsiflorus* [a]

Micro Organism	Inhibition Zone (mm)[b]						MIC (mg/ml)[c]			
	Frac. I	Frac. II	Frac. III	Erac. IV	Ampicillin (40µg/ml)	Nystatin (50IU/ml)	Frac. I	Frac. II	Frac. III	Erac. IV
E. coli	-	10	-	-	19	—-	-	1.824	-	-
P. vulgaris	-	11	-	-	21	—-	-	1.571	-	-
P. aeroginosa	-	11	-	-	17	—-	-	1.652	-	-
B. subtilis	-	15	10	09	21	—-	-	1.415	2.360	2.111
B. pumilus	-	18	11	10	21	—-	-	1.395	2.285	2.245
S. aureus	-	17	10	09	22	—-	-	1.465	2.352	2.254
S. faecalis	-	15	09		22	—-	-	1.523	2.321	-
C. albicans	-	13	-	-	—-	18	ND	ND	ND	ND
A. niger	-	14	-	-	—-	19	ND	ND	ND	ND
A. flavus	-	14	-	-	—-	22	ND	ND	ND	ND
R. oligosporus	-	-	-	-	—-	16	ND	ND	ND	ND

[a] 40µl of solution (50mg/ml) was applied to each well.
[b] Values are mean of five replicates.
[c] Values are mean of five replicates using 5X 10^5 cfu/ml of each culture.
ND- Not Done

Results

Preliminary phytochemical studies

The fraction I has revealed the presence of sterols and triterpenoids, fraction II has tested positive for the presence of flavonoids, fraction III has shown the presence of alkaloids and tannins has been detected in the fraction IV as shown in table no.1.

Antibacterial activity

As table no.2 indicates fraction I has shown no activity either against bacteria or fungi. Fraction II has shown activity against *Escherichia coli, proteus vulgaris, Pseudomonos aeroginosa, Bacillus subtilis, Bacillus pumilus, Staphylococcus aureus* and *Streptococcus faecalis* (Zone of inhibition 10mm, 11mm,11mm,15mm,18mm, 17mm and 15mm respectively). Fraction III has shown activity against *Bacillus subtilis, Bacillus pumilus, Staphylococcus aureus* and *Streptococcus faecalis* (Zone of inhibition 10mm, 11mm, 10mm and 09mm respectively). Fraction IV has shown the inhibitory action against *Bacillus subtilis, Bacillus pumilus, Staphylococcus aureu* (Zone of inhibition 09mm, 10mm and 09mm respectively).

The MIC for fraction II was between 1.824 and 1.395mg/ml. The MIC for fraction III was found between 2.360 and 2.285mg/ml. The MIC for fraction four was between 2.111 and 2.254mg/ml.

Antifungal activity

The fraction II has shown the inhibitory effect on III of the tested fungal strains, *Candida albicans, Aspergillus nigur* and *Aspergillus flavus* with inhibition zone of 13, 14 and 14mm respectively. But the fraction III and fraction IV did not show any activity. None of the fractions were active against *Rhizopus oligosporus.*

Conclusion

Phytomedicines are effective in treating most of the infectious diseases mainly skin infections. Most of the secondary metabolites, serve as plant defense mechanisms against microorganisms, insects and herbivores [11]. The different fractions of methanolic extract of plant *Croton sparsiflorus* found to contain steroids, alkaloids, flavonoids, tannins and resins. The antimicrobial activity of tested medicinal plants can be attributed to any of these constituents. However there was a marked difference in level of activity among these fractions. The results have clearly indicated that fraction two of the methanolic extract of *Croton sparsiflorus* has shown the better antibacterial and antifungal activity than other fractions. This may be due to the presence of flavonoids in the fraction II of the methanolic extract of the plant. Previous reports have indicated that these compounds have shown antibacterial activity [12]. The antibacterial activity of fraction three may be attributed to the presence of alkaloids. Earlier reports have suggested the antibacterial activity of alkaloids [13]. Saponins, which are known to have cytotoxic properties, may be responsible for the antibacterial activity of fraction IV [14]. Hence the detailed phytochemical investigation and antimicrobial screening of secondary metabolites from these plants may yield promising antimicrobial agents.

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Anti-oxidant Activity of Flower Extracts of Thespesia Populnea

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ABSTRACT

Thespesia populnea (TP) is endowed with wide range of pharmacological activities. In continuation of our work on exploring the pharmacological properties of *Thespesia populnea* , in the present work the antioxidant potential of the titled plant is evaluated. Anti-oxidant potential of the plant is evaluated against Superoxide anion, Hydroxyl radical and Reducing power. Titled plant exhibited concentration dependent anti-oxidant activity. Phytochemical studies indicated the presence of flavonoids and phenols in the tested extracts, which might have contributed to anti-oxidant activity. Further studies are in progress to isolate the anti-oxidant moiety of the tested extracts.

KEY WORDS : *Thespesia populnea,* anti-oxidant potential.

Introduction

Oxygen is a vital component for the survival of humans. It is present in the atmosphere as a stable triplet biradical ($^3O_2$) in the ground state. Once it is inhaled it undergoes reduction process and finally metabolizes to water. During this process, a small amount of reactive intermediates such as superoxide anion radicals ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$) and single oxygen ($^1O_2$) are formed [1].These reactive intermediates are collectively called as Reactive Oxygen Species (ROS) [2].ROS can easily initiate the peroxidation of membrane lipids. The lipid peroxides thus formed and their oxidation products such as malondialdehyde (MDA), 4-Hidroxinonenal (4-HNE) are highly reactive , they can react with various biological substrates such as DNA, proteins [3] etc., Apart from this various exogenous reasons such as tobacco smoking, exposure to ionizing radiation, organic solvents and pesticides also play vital role in generation of ROS in the body [4]. ROS are known to be responsible for various diseases like CNS disorders [5], diabetes mellitus [6], liver diseases [7] etc.,

Anti-oxidants interfere with the oxidation process by reacting with free radicals, forming cheelates with free catalytic metals and also by acting as scavengers of oxygen free radicals [8]. Plants are one of the major resources of anti-oxidants, numerous plants are reported to possess anti-oxidant activity [9].

In continuation of our work on evaluation of biological and pharmacological properties of TP [10, 11], in the present work an attempt has been made to evaluate the anti-oxidant potential of TP.

Thespesia populnea **(**Linn**)** soland ex correa (TP) is compact quick growing tree, found along the coastal regions throughout India, also grows as road side tree in tropical regions [12]. Traditionally it is used in scabies, psoriasis, gonorrhea and diabetes [13].

Materials and Method

Plant Material

Flowers of TP were collected from Harapanahalli, Karnataka. The authentication was done by Prof. K. Prabhu, Department of Pharmacognosy, S.C.S. College of Pharmacy, Harapanahalli. A voucher specimen has been deposited at the museum of our college.

Preparation of extracts:

The collected flowers TP were shade dried. The dried flowers were coarse powdered and packed in percolator and percolated with ethanol (70%) and distilled water successively at room temperature. The extracts were concentrated under reduced pressure in Perfit rotary evaporator (bath temperature 50°c) and stored in airtight container in refrigerator below 10°c.

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Preliminary phytochemical screening :

Preliminary phytochemical investigation was carried out on aqueous and 70% ethanol extracts of TP flowers for detection of various phytochemicals by standard methods [14].

Anti-oxidant activity:

Superoxide anion scavenging activity [15]

About 1 ml of Nitroblue tetrazolium (NBT) solution (156µM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (468µM in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of sample solution of ethanolic and aqueous extracts of TP at various concentrations of in water was mixed. The reaction was started by adding 100µl of Phenazine Methosulphate (PMS) solution (60µM PMS in 100mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 minutes and the absorbance at 560 nm was measured against blank.

Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity, % inhibition was calculated by using the following formula :

$$\% \text{ of inhibition} = \frac{\text{Control (absorbance)} - \text{Test (absorbance)}}{\text{Control}} \times 100$$

The results are given in Table-1.

Table No. 1
Superoxide anion scavenging activity of *Thespesia populnea* flower extracts

Group	Absorbance Mean ± SEM	% increase
Control	0.535 ± 0.012	–
Standard 25 µg	0.195 ± 0.003*	63.53%
Aqueous extract 5 µg	0.508 ± 0.004	5.11%
Aqueous extract 10 µg	0.430 ± 0.007*	19.62%
Aqueous extract 25 µg	0.372 ± 0.008*	30.46%
Aqueous extract 50 µg	0.317 ± 0.005*	40.64%
Aqueous extract 100 µg	0.243 ± 0.015*	54.57%
Ethanolic extract 5 µg	0.449 ± 0.004*	16.13%
Ethanolic extract 10 µg	0.413 ± 0.008*	22.85%
Ethanolic extract 25 µg	0.353 ± 0.010*	34.07%
Ethanolic extract 50 µg	0.281 ± 0.004*	47.53%
Ethanolic extract 100 µg	0.216 ± 0.005*	59.67%

Values are the mean ± S.E.M., n=6
*P < 0.001 (vs. Control)
Standard Drug : Sodium metabisulphate

Reducing power [16]

Different concentrations of ethanolic and aqueous extracts of TP flowers were mixed in 1 ml of distilled water so as to get 5 µg, 10 µg, 25 µg, 50 µg and 100 µg / ml concentration. This was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 50°C for 20 minutes. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance (OD) was measured at 700nm.

Increased absorbance of the reaction mixture indicates increase in reducing power. The percentage increase of reducing power was calculated by using the formula mentioned in the estimation of superoxide anion scavenging activity above. The results are showed in Table-2.

Table No. 2
Reducing power activity of *Thespesia populnea* flower extracts

Group	Absorbance Mean ± SEM	% increase
Control	0.118 ± 0.008	–
Standard 25 µg	0.225 ± 0.002***	90.67%
Aqueous extract 5 µg	0.128 ± 0.006	8.47%
Aqueous extract 10 µg	0.148 ± 0.005*	25.42%
Aqueous extract 25 µg	0.176 ± 0.005**	49.15%
Aqueous extract 50 µg	0.205 ± 0.001***	73.72%
Aqueous extract 100 µg	0.218 ± 0.004***	84.74%
Ethanolic extract 5 µg	0.150 ± 0.003*	27.11%
Ethanolic extract 10 µg	0.183 ± 0.005***	55.08%
Ethanolic extract 25 µg	0.195 ± 0.005***	65.25%
Ethanolic extract 50 µg	0.210 ± 0.002***	77.96%
Ethanolic extract 100 µg	0.224 ± 0.002***	89.98%

Values are the mean ± S.E.M., n=6
*P < 0.05, **P < 0.01 and ***P < 0.001 (vs. Control)
Standard Drug : Sodium metabisulphate

Hydroxyl radical scavenging activity [17]

Hydroxyl radical generation by phenylhydrazine has been measured by the 2-deoxyribose degradation, by using the method described by Hathwell and Gutteridge in 50mM phosphte buffer (pH 7.4) containing 1mM deoxyribose, 0.2mM phenylhydrazine hydrochloride and other additions as necessary in a total volume of 1.6ml. Incubation was terminated after 1 hr or 4 hrs and 1 ml each of 2.8% trichloroacetic acid and 1% (w/v) thiobarbituric acid were added to the reaction mixture and heated on water bath for 20 min. The tubes were cooled and absorbance taken at 532 nm.

Decrease in absorbance indicates the increase in the hydroxyl free radical scavenging activity. The percentage reduction in the absorbance is calculated and results are compiled in Table-3.

Table No. 3
Hydroxyl radical scavenging activity of *Thespesia populnea* flower extracts

Group	Absorbance Mean ± SEM (after 1 hr.)	% increase	Absorbance Mean ± SEM (after 4 hr.)	% increase
Control	0.348 ± 0.020	–	0.349 ± 0.011	–
Standard 25 µg	0.116 ± 0.013***	66.64%	0.109 ± 0.008***	68.64%
Aqueous extract 5 µg	0.330 ± 0.006	5.17%	0.324 ± 0.009	7.16%
Aqueous extract 10 µg	0.282 ± 0.005***	18.96%	0.269 ± 0.005*	22.92%
Aqueous extract 25 µg	0.241 ± 0.005***	30.76%	0.224 ± 0.006***	35.81%
Aqueous extract 50 µg	0.226 ± 0.010***	35.26%	0.204 ± 0.005***	41.54%
Aqueous extract 100 µg	0.135 ± 0.003***	61.20%	0.126 ± 0.008***	63.89%
Ethanolic extract 5 µg	0.301 ± 0.004*	13.50%	0.270 ± 0.009*	22.76%
Ethanolic extract 10 µg	0.276 ± 0.006***	20.68%	0.239 ± 0.003***	31.51%
Ethanolic extract 25 µg	0.221 ± 0.005***	36.49%	0.201 ± 0.004***	42.40%
Ethanolic extract 50 µg	0.186 ± 0.010***	46.55%	0.171 ± 0.015***	51.00%
Ethanolic extract 100 µg	0.125 ± 0.005***	64.08%	0.119 ± 0.010***	65.90%

Values are the mean ± S.E.M., n=6
*P < 0.05 and ***P < 0.001 (vs. Control)
Standard Drug : Sodium metabisulphate

Results

Preliminary phytochemical studies of the extracts showed the presence of flavonoids and phenols. Ethanolic extract of TP flowers demonstrated anti-oxidant activity in superoxide anion scavenging activity, reducing power and hydroxyl radical scavenging activity models by concentration dependant manner. Similar effect was also seen with aqueous extract of TP. The anti-oxidant potential of the aqueous and ethanolic extracts of titled plant was almost equipotent to that of standard at 100µg concentration.

Discussion

In PMS/NADH-NBT system, superoxide anion generated by dissolving oxygen by PMS/NADH coupling reaction reduces NBT. Thus the decrease in absorbance at 560 nm indicates consumption of superoxide anion in the reaction mixture. Results from the present study indicate extracts of TP showed superoxide anion scavenging activity in concentration dependent manner. For the measurement of reductive ability, we employed the Fe^{3+} - Fe^{2+} transformation in the presence of aqueous and ethanolic extracts of TP. The reducing power of extracts of TP increased with increasing the amount of sample. In biochemical systems, superoxide radical and H_2O_2 react together to form the hydroxyl radical OH. Hydroxyl radical generation by phenylhydrazine has been measured by the 2-deoxyribose degradation. In the hydroxyl radical scavenging activity also the tested extracts showed concentration dependent pattern.

Flavonoids and phenols are one of the very important plant constituents. Many plants containing flavonoids and phenols are reported to possess antioxidant activity [15,18]. Since phytochemical study of extracts showed the presence of flavonoids and phenols , antioxidant activity of the TP may be attributed to these constitutents. Further studies are in progress isolation and identification of the antioxidant component of the plant.

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Antilithiatic activity of ethanolic extracts of *Hibiscus rosa sinensis* linn in albino rats

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ABSTRACT

In the indigenous system of medicine many plants have been found to be useful in the treatment of urinary calculi among these mucilaginous infusion of *Hibiscus rosa sinensis* Linn used as a antilithiatic agent.The ayurvedic and folk medicinal practitioners of undivided Dakshina kannada and northern kerala recommend a particular variety of *Hibiscus rosa sinensis* linn ,locally known as *Dasavala*.The study was investigated the effect of *Hibiscus rosa sinensis* linn which is proved to be antilithiatic activity .The study was conducted on experimentally induced calcium oxalates stones by using sodium glycollate 3% in albino rats. A three dosage of 150,200,250 mg/kg bodyweight was carried out.Hypercalcuria hyperoxaluria are the parameters considered in present study. *Hibiscus rosa sinensis* Linn ethanolic extract at three dosage level 150,200,250 mg/kg body weight p.o were shown a significant $p< 0.001$ antilithiatic activity by reducing the out put urinary electrolytes of calcium and oxalates. In the present study the effect was compared with standard ayurvedic formulation Cystone [R] at a three dosage level 150,200,250 mg/kg body weight p.o the levels of excretion of calcium and oxalates in 24hrs urine sample after treatment were compared with control group there exists a significant difference $p<0.001$ between the groups in the excretion of urinary electrolytes calcium and oxalates and *Hibiscus rosa sinensis* Linn has significant antilithiatic activity when compared with standard ayurvedic formulation Cystone [R] thus claiming the claim made in the indigenous system of medicine.

KEY WORDS: Calculi, Antilithiatic agent, Cystone

Introduction

Traditional system of medicine remains the major source of healthcare for more than 2/3 of the world's population. The plant kingdom is the most fruitful ground for the discovery of drugs for those ailments for which there is no specific treatment in the modern system of medicine at a lesser cost with negligible harm to man kind [3] In the indigenous system of medicine many plants were found to be useful in the treatment of urinary calculi. Among this mucilaginous infusion of *Hibiscus rosa sinensis* Linn is used as an antilithiatic agent [7.8]. The ayurvedic and folk medicinal practitioners of dakshinakannada and northern Kerala recommend a particular variety of *Hibiscus rosa sinensis* Linn, locally known as a *dasavala* which is claimed to be effective in the treatment of urinary calculi. The present study was planned to assess the efficacy of ethanolic extract of *Hibiscus rosa sinensis* Linn in the prevention and management of kidney stones in sodium glycollate induced urolithiasis in the urinary tract and kidney of rats.

Kidney stones are one of the oldest diseases known to mankind and yet witnessed in this century. Depending upon the size of calculi smaller calculi made up of coarse and small particles appear as"*Gravel*" Eg: uric acid stones, cysteine stone, calcium oxalate stone, and they arise in kidney or pelvic region of kidney. Larger calculi appear as "*staghorn*" Eg:Struvitestones. They arise in entire portion of kidney. Calculi which contain single constituent is termed as simple stones Eg:uricacid.cystine stones. Calculi containing two or more constituents is termed as compound calculi Eg:calciumoxalate and struvite stones [4,9].

There is a persistent interest towards the alternative remedies from the plant sources a large number of plants were screened for this purposes in India and abroad for antiurolithiatic activity, flowers of *Hibiscus rosa sinensis* Linn is one such plant having tremendous therapeutic applications but not exploited as a medicinal plant so far.

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Hibiscus rosa- sinensis Linn is a shrub of family *Malvaceae* a native of South Eastern Asia very commonly cultivated and relatively old habituation and cultivation in wide range of situations now commonly found throughout the tropics as a house hold plant throughout the world every part of the shrub has been used as a traditional medicine and as a house hold remedy against various ailments [3, 11].

The wide spread of this shrub in India and other countries and also keeping view of pharmacological activity *Hibiscus rosa sinensis* Linn makes it an attractive candidate drug for future pre-clinical and clinical research.

Material and Methods

The plant material was identified and authenticated by Mrs B.Ushanalini Prof and HOD Department of Botany, St Agnes College, Mangalore.

Preparation of *Hibiscus rosa sinensis* Linn extract

Method: Solvent Extraction using maceration process [3]

The fresh flowers of *Hibiscus rosa sinensis* linn were collected from locally available gardens it was authenticated by the competent botanist at department of Botany, St Agnes College, Mangalore. The fresh flowers were subjected for solvent extraction methods of drug extraction by maceration process. 25%v/v ethanol were used during the process of drug extraction. Maceration was carried out for duration of 48 hrs. Filtrate was collected and it was subjected for dryness under controlled temperature in warm water bath at temperature 55^0C. Mucilaginous extract was obtained were collected and it was reconstituted with distilled water and used for the experiment.

Animal selection

Adult male albino rats of *Wister* strain of weighing between 100-120gms were bread in central animal house the animal room was well ventilated, and no special arrangement were made for heating and cooling animal were given normal rat feed and water *adlibitum* for a week [4].

Diet

The rat feed was supplied by Hindustan lever ltd Bombay. The pelleted feed contains protien 21%, Lipids 5%, Crudefibre 4%, Ash 8%, Calcium 1%, Phosphorous 0.6%, Nitrogen free extract 55% and the diet was enriched by vitamin and trace elements

Acute toxicity test and selection of dose:

Albino mice of either sex weighing 20-25 gms and 90 days age were used to determine LD 50 of the drug extract. The gum acacia 2% was used as a vehicle to suspend the various fractions and were administered intra peritonealy. The hydro alcoholic extract and its various fractions were found to be non toxic up to 2500mg/kg/b.w. Hence 1/10th of lethal dose were taken as maximum therapeutic dose for subsequent studies [10].

Preliminary Investigations

Rats were hydrated for 24hrs and urine samples of 24hrs were collected by using individual metabolic cages and subjected were analyzed for physical, chemical and normal excretion of calcium and oxalates were also estimated [1,10].

Preliminary Phyto chemical studies

Fractions of drug extract were subjected to qualitative chemical investigation for identification of phytoconstituents [4,6] Citric acid, flavonoids carbohydrates, cynogenic glycosides, tartaric acid.

Induction of calcium oxalate stones.

Calcium oxalates is the common constituent of urinary stones. Calcium oxalate stone were induced in rats by the method *chow et al* [2,5] using sodium glycollate 3% in saline 0.9% at a dosage of 3ml/daily for 4 weeks by oral route. After 4 weeks the calcium oxalates were formed. This was detected by physical examination the formation of calcium oxalates in the bladder or kidney of the rats. Quantitative estimation of calcium and oxalates were carried out and calcium oxalate crystals.

Experimental Design

Group 1 (Control group)

Normal estimation of urine electrolytes were carried out Sodium glycollate 3% in 0.9% saline were administrated for 4 weeks and at the end of 4^{th} week calcium oxalate stone were formed were confirmed by specific tests. And this experiment was carried out for another 4 weeks till 8^{th} week and the formation of calcium oxalate stones in control group was confirmed by physically by dissecting the bladder of rat, chemical estimation of calcium and oxalates are also confirmed the increased out put of calcium and oxalates.

Group 2 *Hibiscus rosa sinensis* linn extract treated group:

Sodium glycollate at dosage of 3% in 0.9% saline was administered for three different groups for a duration of 4 weeks and at the end of 4 week sodium glycollate3% formation of calcium oxalate was confirmed and *Hibiscus rosa sinensis* Linn extract at three different dosage 150, 200 and 250 mg/kg body weight were given for another 4 weeks at the end of 8^{th} decreased out put of calcium & oxalates were observed

Group3 Cystone® treated group:

Sodium glycollate at dosage of 3% in 0.9%saline was administered for three different groups for a duration of 4 weeks and at the end of 4 week sodium glycollate 3%

formation of calcium oxalate was confirmed and Cystone® at three different dosage 150, 200 and 250 mg/kg body weight were given for another 4 weeks at the end of 8th decreased out put of calcium & oxalates were observed.

Results and Discussion

In control group the changes in physical parameter [4, 6, 9] viz, colour, P^H, volume and appearance, , the urinary clearance of calcium and oxalate were increased significantly (P<0.001) when compared to the normal urine. In *Hibiscus rosa sinensis* Linn extract treated group there exists a significant (P<0.001) decrease in the clearance of urinary calcium and oxalates at the end of 8th week. In cystone® treated group there exists a highly significant decrease in the clearance of urinary calcium and oxalate at the end of 8th week (Table 1). Flowers of *Hibiscus rosa-sinensis* Linn has a diuretic action and also it contains, stone inhibiting factors viz; citrates and tartaric acid prevents the formation of kidney stones hence drug extract facilitates the dissolution of preformed stones acting as antihypercalciuric and anti hyperoxaluric agent and thus proving the claim made in the indigenous system of medicine.

Conclusion

The present study was under taken to assess the antilithiatic activity of the *Hibiscus rosa sinensis* Linn in comparison with Cystone® *Hibiscus rosa sinensis* Linn ethanolic extract significantly decrease in the etiopathogenic factors of stone formation. The mechanism of inhibition of stone formation by *Hibiscus rosa sinensis* Linn is therefore thought to occur through the restoration of risk factors in the urine by reducing the levels of calcium and oxalate and increasing the level of inhibitors of stone formation.

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Table 1

Antilithiatic activity of ethanolic extracts of *Hibiscus rosa sinensis* linn in albino rats

Dosage			0 DAY	4th Week	8th Week
			Mg/dl	Mg/dl	Mg/dl
Group I Control		Calcium	5.38 ± 0.18	13.65± 0.49	18.3 ± 0.90
		Oxalate	4.6 ± 0.10	17 ± o.90	17.5 ± 1.24
Group II	150mg/kg	Calcium	5.4 ± 0.17	14.2 ± 0.51	17.1±0.85*
		Oxalate	4.8 ± 0.15	18 ± 0.91	16.5 ± 0.95*
Sodium + *Hibiscus rosa sinensis* Linn extract treatment	200 mg/kg	Calcium	5.3 ± 0.15	14.5 ± 0.55	14.5 ± 0.8*
		Oxalate	4.8 ± 0.15	16.5 ± 0.85	13.1 ± 0.68*
	250 mg/kg	Calcium	5.3 ± 0.68	19.0 ± 0.73	12.3 ± 0.5**
		Oxalate	4.8 ± 0.57	18.6 0.56	10.5 ± 0.5**
Cystone (R) treatment	150 mg/kg	Calcium	5.4 ± 0.69	18.5 ± 0.78	14.1 ± 0.68*
		Oxalate	4.9 ± 0.58	19.1 ± 0.76	12.8 ± 0.865*
	200 mg/kg	Calcium	5.5 ± 0.81	14.5 ± 0.91	11.3 ± 0.865*
		Oxalate	4.6 ± 0.91	13.8 ± 0.58	10.8 ± 0.71*
	250 mg/kg	Calcium	5.1 ± 0.25	17.5 ± 0.76	9.3 ± 0.76**
		Oxalate	4.3 ± 0.31	18.3 ± 0.79	8.5 ± 1.23**

Mean ± SEM, n = 6, ** p<.001, * p<.01

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Bioelectro Analytical Study of Colon-Specific Prodrug Using Modified Carbon Paste Electrode

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ABSTRACT

Trametus *versicolor* immobilized carbon paste electrode was developed for the detection of sulfa drugs. The bioelectroanalytical properties of a colon-targeted prodrug of 5-aminosalicylic acid (5-ASA), salicylazosulphapyridine, (sulfasalazine) was investigated in aqueous solutions at modified carbon electrodes using cyclic voltammetry. It was found that these compounds can be both reduced and oxidised at a modified carbon electrode. The reduction and oxidation potentials of the compounds were dependent on the pH and the structure of the compounds. In an acidic media substrate is reduced in a 4e (-)/4H (+) process yielding 5-ASA and sulfanilic acid. In neutral and weakly basic media sulfasalazine is reduced in 2e(-)/2H(+) process resulting in the hydrazo intermediate that is stable enough to enable its reoxidation back to substrate in the time scale of the cyclic voltammetry. A further electrochemical characterization of the formed reduction products aminosalicylic acid was also carried out. The optimum response was realized by electrode constructed using 9 mg of dry cell weight per 1 g of carbon paste and operating at 28 ^{0}C, pH 6.8, phosphate buffer. Operating at these optimum conditions the biosensor had excellent selectivity against the substrate. The peak current varied linearly with increase in drug concentration. The proposed biobased electrochemical sensor is a sensitive, simple, inexpensive and user-friendly analytical method for the detection of colon-specific azo prodrugs.

KEY-WORDS: Trametus *versicolor; Bioelectroanalytical*; Sulfasalazine; carbon paste electrode: Cyclic voltametry.

INTRODUCTION

A biobased electrochemical sensor is presented in this paper, as a rapid, sensitive, simple, inexpensive and user-friendly analytical method against to classical assays for the detection of sulfonamide antibiotics samples. During recent decades the use of antibiotics and chemotherapeutics has increased considerably. Among the different kind of antibiotics, sulfonamides are widely used because of their broad spectrum of antibacterial activity to treat infections and to preventively control the outbreak of diseases, to improve feed efficiency and to promote growth in animals [1]. Estimation of sulfa drugs in various biological samples stress for a simple and sensitive analytical technique. The presence of certain antimicrobial agent residuals in environment/ food matrices constitutes a potential hazard and may cause allergic reactions [2]. As a consequence, food and environmental regulatory agencies have established control programs due to the increasing concern about the possibility that residues of antibiotic arrive to the consumer through food of animal origin and through the industrial effluents discharge [3]. Routine methods used for determination of antimicrobials are usually based on growth inhibition of a sensitive test organism [4]. Microbial assays detect a broad spectrum of antibiotics and chemotherapeutics but their sensitivity to sulfonamides are usually non-satisfactory. The chromatographic techniques, involving extraction and extensive purification procedures, are also used to confirm in positive findings. As a consequence, the development of rapid, inexpensive, sensitive and on-site analytical strategies which can be used in a wide variety of biological and environmental samples are required, since the standard chromatographic technique does not meet these requirements. Electrochemical sensors have revolutionized modern analysis because of their technical simplicity and speed in response by the direct transduction to electronic equipments [5]. Mass fabrication, low cost and decentralized on-site/in-field analysis are other important features of electrochemical sensors to be considered as analytical tool. This approach has been successfully utilized for sulfasalazine analysis in the present study. It is an azo conjugate of 5-ASA and

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sulfapyridine. The active moiety is 5-ASA, whereas sulfapyridine acts as a carrier that protects 5-ASA from the acidic pH of stomach, and prevents its absorption from small intestine, delivering it to colon [6].

Aromatic azo compounds constitute a very important class of organic compounds [7] because of their widespread applications in many areas of technology and medicine [8]. They are well known for their use as analytical reagents, in dye industry or as chemotherapeutic drugs [9,10]. The use of laccase in the textile industry is growing very fast, since besides to decolourise textile effluents, laccase is used to bleach textiles and even to synthetise dyes [11]. The laccase produced by *T. versicolor* can be used to decolorize and detoxify a variety of recalcitrant xenobiotics including: textile dyes, polychlorinated biphenyls, and polyaromatic hydrocarbons [12, 13]. Thus *T. versicolor* was selected biorecognition element in the biosensing element preparation.

Materials and Methods

Chemicals and Reagents

Sulfasalazine was obtained as a gift sample from Wallace Pharmaceutical Pvt. Ltd., Goa K_2HPO_4, KH_2PO_4, $MgSO_4$,$FeSO_4$, Na_2HPO_4, NaH_2PO_4, graphite powder and mineral oil (white, light) were purchased from Fisher Scientific. Potato dextrose agar medium was obtained from Hi Media, India. All the chemicals used were of analytical grade unless otherwise stated. All solutions were prepared by double distilled water. Phosphate buffer (0.05 M, pH 6.8) was served as supporting electrolyte. All solutions were prepared with deionized water.

Culturing of white-rot fungi and determination of enzyme activity.

The white-rot fungi, Trametes *versicolor* (ATCC – 20869, NCIM No- 1086). Culture was obtained from National Collection of Industrial Microorganisms (NCIM), Pune. The strain was maintained on potato/dextrose agar plates, by serial transfers and kept on agar slants at 4 ^{o}C. Subcultured every month

Apparatus

Experiments were performed with the Amperometric Biosensor Detector. Electrochemical measurements were performed with an Auto lab PGSTAT30 (Ecochemie) potentiostat/ galvanostat running under the GPES 4.7 software connected to a X-Y-t recorder model. All potentials are referred to saturated calomel electrode (SCE) from BAS (Bioanalytical Systems), while platinum was employed as the auxiliary electrode. The working electrodes were modified carbon electrode (m-CPE) [14,15]. The electrodes were inserted into the cell through its Teflon cover. Before each experiment electrodes were rinsed with water and dried. The experiments were performed in phosphate buffer solution pH 6.8. Scans rate was fixed to 20mV/s by initial scanning through different scan rates. All experiments were carried out at room temperature.

Construction of modified–carbon paste electrode (m-CPE) electrodes

The working carbon paste electrode was prepared by using appropriate weight of cells, 1g of carbon paste containing 75% (w/w) graphite powder and 25% (w/w) mineral oil and hand-mixing of carbon particles with microbes and mineral oil [16,17]. The whole cell modified carbon paste was subsequently packed firmly into the electrode cavity (3mm diameter ×10mm depth) of a hollow borosilicate glass tube. Electrical contact was established via a copper wire. Before each use, the electrode surface of m-CPE electrodes were renewed by a simple polishing procedure, wetted with double distilled water and then thoroughly smoothed with abrasive paper and then with alumina paper [18].

SEM characterization of modified–carbon paste electrode

The scanning electron microscopy technique was used to evaluate the distribution of the trametes *versicolor* whole cell on the surface of the m-CPE. For this purpose, after the capture of the cells by the CP electrode, fig.1. (i, ii, iii, iv) SEM was performed. The same acceleration voltage (15 kV) but different resolutions 100 and 500 μm were used.

Results and Discussion

SEM characterization of modified–carbon paste electrode

The scanning electron microscopy technique was used to provide qualitative information about size and distribution of whole cells on the surface of the m-CPE. As can be seen in the picture (0.5 mm Fig. i, 100μm Fig. ii) the cells are irregularly distributed on the surface of the electrode. Thick areas showing a higher concentration of cells (Fig. iv) are followed by almost bare areas where the transducer can be clearly seen (Fig. iii), This distribution is beneficial since the bare areas of CPE transducer are easily reached by the soluble mediator in order to obtain the rapid electrochemical signal.

Optimization of operational conditions

The parameters of applied potential, amount of biocatalyst, operating pH and operating temperature affecting the response of microbial biosensor were optimized using $3.0 \times 10\text{-}5$ M sulfasalazine.

Hydrodynamic voltammogram

A review of the sulfa drug microbial degradation/ reduction pathway indicates the presence of intermediates hydrazo and aromatic amine, compounds that are known to

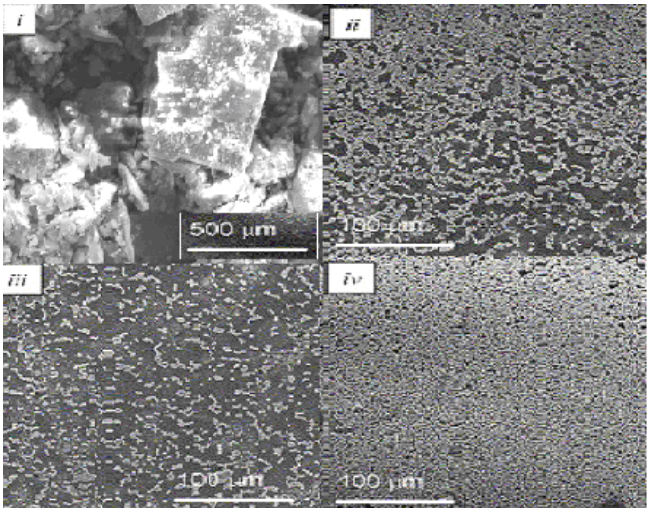

Fig.1. Scanning electron microphotographs showing the distribution of microbial cells.

Figure 1 indicates the Scanning electron microphotographs of distribution of microbial cells on the surface of CPE sensors taken at 0.5 mm (i) and 100 ìm (ii–iv) of resolution. Identical acceleration voltage (15 kV) was used in all cases.

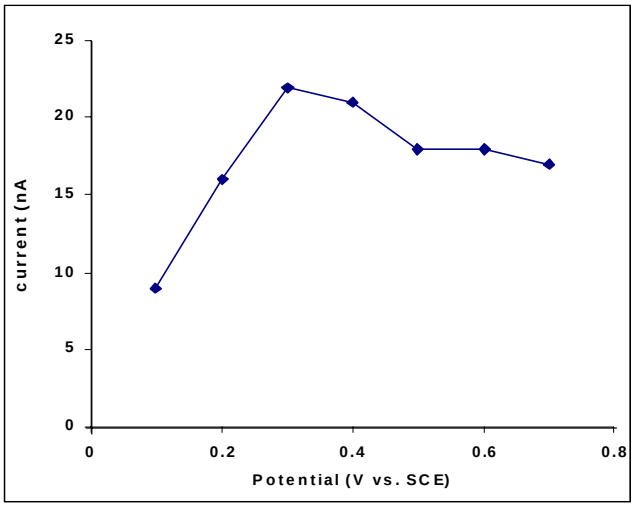

Fig.2. Hydrodynamic voltammogram

Figure 2 showing the hydrodynamic voltammogram for 0.01mM sulfasalazine at the carbon paste electrode in 50 mM, pH 6.8, phosphate buffer at 28 ^{0}C. Data are given as mean±1S.D. for three measurements.

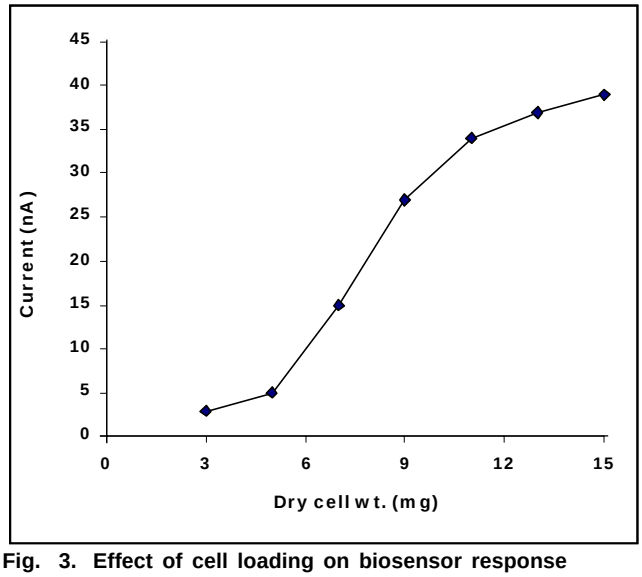

Fig. 3. Effect of cell loading on biosensor response

Figure 3 Effect of cell loading on biosensor response to 0.04mM sulfasalazine in 50mM phosphate buffer, pH 6.8, at 30 ^{0}C. Data are given as mean±S.D for three measurements.

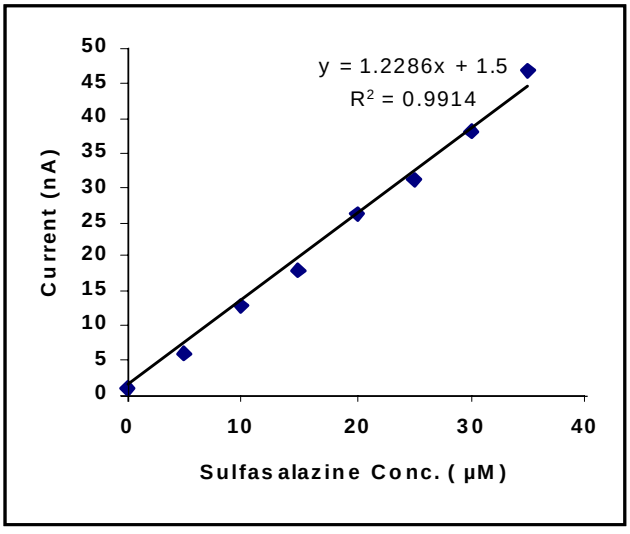

Fig.4. Calibration graph for sulfasalazine at microbial biosensor

Figure 4 indicating calibration graph for sulfasalazine at prepared microbial biosensor, in 50mM, pH 6.8, phosphate buffer at 28^0 C with 9 mg cell loading. Data are given as mean ± S.D. for three measurements.

be electroactive [19]. The former is an oxidized compound and it can undergo reoxidation back to original compound. While the later is a reduced compound (major compound at acidic and neutral range). The electro reduction current corresponding to the amine compound increased as a function of applied potential to reach a maximum at 270mV (versus SCE) and plateaued. The potential of 0.27 V was used in the subsequent studies.

Effect of microbial cell loading

The composition of the prepared microbial based electrode has a profound effect upon detection limits. Figure 2 examines the influence of the electrode composition upon the resulting current values. The composition containing less than 6 mg microbial cells, has narrow linear range. On the other hand, microbial cells amount more than 9 mg has lower sensitivity. Hence, 9 mg was chosen as optimum amount considering linear range and facility in electrode preparation. Increasing the cell loading beyond 13 mg, however, increased the background current and also the signal was very noisy. Therefore, 9 mg of dry cell weight per 1 g of carbon paste was selected for subsequent experiments.

Effect of preadaptation of cells using sulfa drugs

Experiments revealed that the bioconversion of sulfa drugs in Trametus *versicolor* sp. is enhanced by pre-exposure of cells to sulfa drug, indicating inducible gene expression of the enzymes involved in sulfa drug catabolism. However, there is no report on the relationship between the adaptation period and the degree of the activity enhancement.

To investigate preadaptation effect, culture medium was supplemented with 250 ppm of sulfa drug in a 5 consecutive cycles. To determine biosensor response, electrodes were prepared using constant amount pre-exposured cells (9 mg of cells /gram of carbon paste) harvested after different incubation cycles. Results were as shown in Table 1, biosensor response increased after the first adaptation period on sulfa drug and reached a plateau after five adaptation cycles. Additionally, the time required for the cells to consume most of the added sulfa drug (indicated by the disappearance of the color and by colorimetric readings) shortened from 15 to 5 h. Cells generated after five repeated additions of Sulfa drug were used for biosensor construction. Addition of sulfa drug to the culture did not affect the growth of the cells as indicated in the cell density values.

Effect of pH

The catalytic activities of the enzymes involved in substrate catabolism are a function of the pH. According to the optimization studies, the effect of pH on the electrode response was investigated by using phosphate buffer systems (50 mM) between pH 5.0 and 8.5 The response current of the bioelectrode to substrate increases significantly from pH 6.0 to 6.8, and then a sharp decrease is obtained at pH values higher than 8.5. As a result pH 6.8 was chosen as optimum pH for further studies.

Effect of temperature

The amperometric response of the microbial electrode to 1.0 × 10-5 M sulfa drug was measured at different temperatures varying from 20 to 35 °C. From 20 to 30 °C, the peak current increases with temperature till 30 °C and stay almost constant up to 35 °C. Above that temperature, a decrease in the amperometric response was observed. This decrease can be attributed to the thermal deactivation of the enzyme at higher temperatures. Moreover, thermal stability of the microbial cells at 25, 30 and 35 °C was tested in previously was found that the cells is more stable at 25 °C compared to other higher temperatures. Hence, temperature around 25^0C was used for all subsequent experiments.

Analytical characteristics of the microbial electrode

Calibration

The calibration plot generated using the optimum conditions determined above (pH 6.8, 0.05M phosphate buffer, 9mg cell loading per 1 g of carbon paste and working electrode poised at 0.27 V) is shown in Fig. 4(the plot was prepared from the sensor steady-state response data). The biosensor response was linear up to 40µM with a sensitivity (slope) of 1.22 nA per µM sulfasalazine ($r2$ = 0.991). At higher concentrations, standard curve showed a deviation from linearity.

Conclusions

Detection of sulfasalazine using the microbial electrodes shows several advantages over other analytical methods. A microbial based electrode configuration relies on the biochemical recognition and the electrochemical transduction. An amperometric biosensor for sulfasalazine is described, which was prepared by microbial modified CPE. The attractive properties and behaviors of CPE as the composite materials enable us to obtain sensitive biosensing systems. The bioelectrode displayed a rapid and sensitive response to sulfasalazine. Only a reduction wave appeared with peak potential $E_{p,c}$ -0.270 V on cathodic scan and an oxidation wave with peak potential $E_{p,a}$ -0.021 V on reverse scan. The amperometric response current displays a linear relationship with increase in the concentrations of drug.

Table 1
Effect Of Trametus *Versicolor* Adaptation Time On Sulfasalazine

Adaptation time (h)	Biosensor response to 30µM sulfasazine (nA)	Cell density (A_{600})
0	19	0.89±0.03
15	22	0.87±0.02
12	25	0.93±0.03
8	30	1.10±0.04
6	34	0.88±0.01
5	36	0.91±0.02

Data are given as mean ± S.D for three measurements.

The sensitivity of the developed system to sulfa drug was found to be good. This very simple and low cost sensor does not require trained personnel and expensive instrument. These features make it a potentially attractive analytical tool for sulfa drug analysis. On the other hand, the proposed system could be a good example for the utilities of modified carbon paste biosensors in electro analysis due to its low cost, comparable sensitivity and ease in the fabrication.

Acknowledgments

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Synthesis and Antidepressant activity of Venlafaxine and its derivatives

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ABSTRACT

Venlafaxine and its derivatives were synthesized and screened for anti depressant activity on mice through forced swim test model (FST Model). All the derivatives showed significant antidepressant activity.

KEY WORDS: antidepressant, venlafaxine.

Introduction

Venlafaxine is a dual reuptake inhibitor that combines the action of both serotonin reuptake inhibitor (SRI) and nor-epinephrine reuptake inhibitor (NRI) with diminished alpha, antihistamine and anti-cholinergic liability [1]. John P. Yardely etal [2] had reported the synthesis of Venlafaxine by two methods, method-1 involves substituted phenylacetonitrile was condensed with cyclohexanone at -70°C under nitrogen, catalytic hydrogenation of β-hydroxynitrile with rhodium on alumina catalyst yielded primary amine. N-methylation was accompanied by Eschweiler-clark procedure. Method 2 involves substituted phenyl acetic acid was first converted to required amide, the amide was then condensed with cycloalkanone. β-hydroxynitrile is reduced to primary amine using either aluminium hydride [3] or borane/THF complex [4] .The present research work involves p-methoxy acteo nitrile is condensed with cyclohexanone in presence of TBAB at 0-5°C to afford intermediate which upon hydrogenation with Raney Nickel yielded primary amine. N-methylation was accomplished by Eschweiler-clark procedure. Primary amine is used as starting material for the synthesis of various Venlafaxine derivatives. All the compounds of the series have been screened for their antidepressant activity, advantage of present proposed research work was cost effective as compared to the above methods.

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Experimental

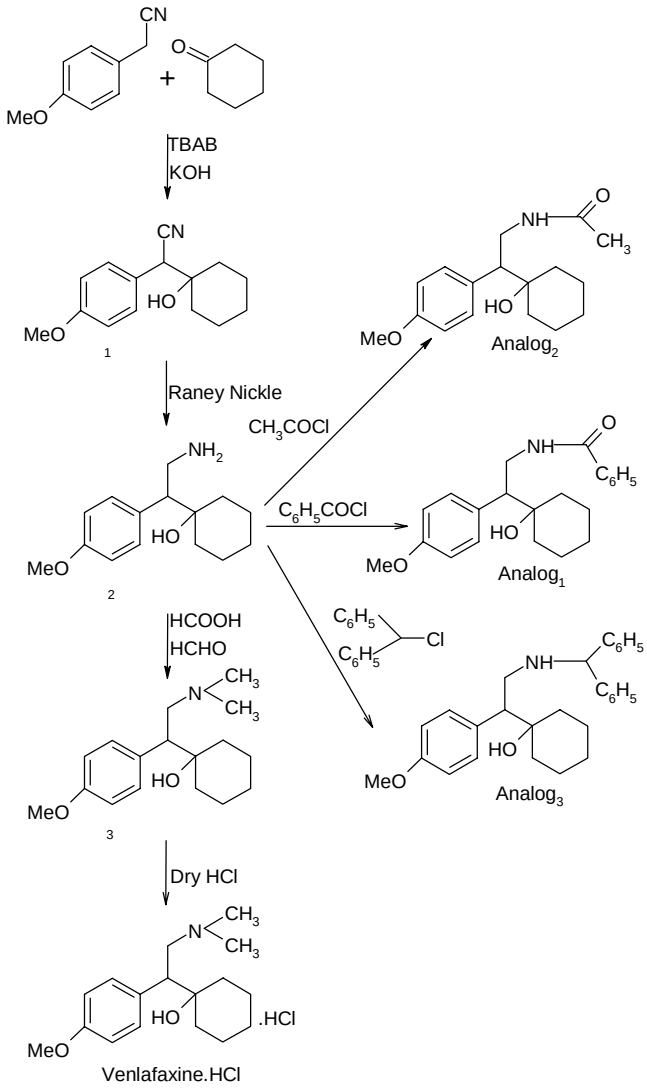
Scheme – I:Synthesis of 1- [Cyano (4-methoxy phenyl) methyl] cyclohexanol. (1)

Potassium hydroxide (0.071M) and Tetra butyl ammonium bromide (TBAB) (0.007M) was dissolved in 40 mL of methanol and was cooled to 0-5°C, then this mixture of p-methoxy phenyl acetonitrile (0.1M) and cyclohexanone (0.1M) were added drop wise for a period of half an hour with vigorous stirring.Then the reaction mixture was vigorously stirred at 0-5°C, for 2 hours. The white crystalline product obtained was filtered and washed several times with water until the filtrate is neutral to red litmus paper and the product is recrystallised from methanol and dried. **(1)**, m.p.120-122°C, yield (51.46%). **IR**; 3409(OH), 2250(CN), 1253,1033(C-O-C) cm^{-1}; **^{1}H NMR** ($CDCl_3$) ; δ 7.25 and 6.9 (q, 4H,ArH),3.8 (s,3H,OCH_3), 3.72 (s, 1H,CHCN), 1.4- 1.8 (m, 10H,Cyclohexyl H).

Synthesis of 1-[2- Amino-1- (4-methoxyphenyl) ethyl] cyclohexanol. (2)

Compound **(1)** (0.08M) was dissolved in 300 mL of methanol in hydrogenator chamber. To this triethylamine (0.013M) was added and the reaction mixture was hydrogenated over Raney Nickel at 50-60° C for 4-5 hours, after the reaction is complete the catalyst was filtered. The filtrate was distilled to collect the oil, to this 50 ml of water was added and the solution was made pH-4 with acetic acid, then the aqueous layer was washed with

Scheme-1:



toluene for 2 to 3 times. The pH of the aqueous layer was adjusted to 8 with solid Na_2CO_3, the product formed is extracted with ethyl acetate. The ethyl acetate was distilled to get the product **(2)**, yield (53.97 %) and oil was dissolved in acetone and acidified to pH- 2 by passing dry HCl gas at 0-5° C to precipitate as HCl salt as crystalline solid, m.p. 166-168°C, yield (59%). **IR**; 3400-3200 (NH_2 and OH), 1247,1035 (C-O-C) cm^{-1}; **^{1}H NMR** ($CDCl_3$); δ 9.56(s, (b),H,HCl), 7.15 and 6.85 (d,d,4H,ArH),7.5 (s,3H,OCH_3), 3.35 (m,1H,$CHCH_2NH_2$), 3.15 (m,2H,CH_2NH_2), 1.25-1.95 (m,10H,Cyclohexyl H).

Synthesis of 1-[2- (Dimethylamino)-1-(4- methoxy phenyl) ethyl] cyclohexanol. (Synthesized Venlafaxine). (3)

Compound **(2)** (0.04M), Formaldehyde (0.12M), Formic acid (0.25M) and 100ml of water were taken in round bottom flask and the solution was refluxed over night with stirring. After the reaction is complete the solution was made pH-2 with concentrated HCl and extracted with ethyl acetate to remove pink colored impurity. The solution was basified with 50% NaOH, extracted with ethyl acetate. The ethyl acetate was distilled to get the product **(3)**, yield (75%) and oil was dissolved in ethyl acetate and acidified to pH-2 by passing dry HCl gas at 0-5°C to precipitate as HCl salt as crystalline solid, m.p.212-214°C, yield 70.07%. **IR**; 3387(OH), 1247,1032(C-O-C) cm^{-1}; **^{1}H NMR** ($CDCl_3$); δ 11.7(s, (b), H,HCl), 7.13 and 6.85 (d, d,4H,ArH), 3.76 (s, 3H,OCH_3), 3.35 (t, 2H, $CH_2N(CH_3)_2$), 3.15(m, H,$CHCH_2N(CH_3)_2$), 2.62 and 2.82 (s,s, 6H,$N(CH_3)_2$), 1.2-1.95 (m, 10H,Cyclohexyl H) ; **ms**; m/z 278.2 (M^+ +1).

Synthesis of 1-[2- (benzamido)-1-(4- methoxy phenyl) ethyl] cyclohexanol. Analog-1.

Compound **2** (0.008M) was dissolved in pyridine (12 mL). The solution was cooled to 0-5ºC over ice bath and benzoyl chloride (0.008M) was added drop wise with stirring. Then the reaction mixture was stirred for 6 hrs at room temperature, and was added to water, acidified with dilute HCl to neutralize pyridine and then extracted with ethyl acetate. Then ethyl acetate layer was separated and distilled to collect the solid sample, which was recrystallised from petroleum ether, **Analog-1**, m.p.200-202°C, yield 53%. **IR**; 3400-3300 (OH and NH), 1660 (C=0), 1247,1033 (C-O-C) cm^{-1}; **^{1}H NMR** ($CDCl_3$); δ6.85,7.24,7.34,7.56 (d,m,d,9H,ArH), 3.81(s,3H,OCH_3), 6.34 (s (b), 1H,CH_2NH), 3.65 (m,2H,$CHCH_2$), 2.95(m,1H,$CHCH_2$), 1.56 (m,10H,Cyclohexyl H).

Synthesis of 1-[2- (acetamido)-1-(4- methoxy phenyl) ethyl] cyclohexanol. Analog-2.

Compound **2** (0.008M) was dissolved in 12mL of pyridine. The solution was cooled to 0-5ºC over ice bath, add acetyl chloride (0.008M) was added drop wise with stirring. Then the reaction mixture was stirred for 3 hours at room temperature, and was added to water, acidified with dilute HCl to neutralize pyridine, then extracted with ethyl acetate and ethyl acetate layer was separated and distilled to collect the solid sample, **Analog-2**, m.p.94-96°C, yield (55.79 %.). **IR**; 3400- 3300 (OH and NH), 1651 (C=0), 1248,1035 (C-O-C) cm^{-1}; **^{1}H NMR** ($CDCl_3$); δ7.16 and 6.85 (d,d,4H,ArH), 5.38 (s,1H,CH_2NH), 3.8 (s,3H,OCH_3), 3.44 (m,2H,CH_2NH), 2.75 (m,1H,CH_2CH), 1.8 (s,3H,$NHCOCH_3$), 1.48 (m,10H,Cyclohexyl H).

Synthesis of 1-[2-(Benzhydryl-amino)-1-(4-methoxy-phenyl) ethyl] Cyclohexanol. Analog-3.

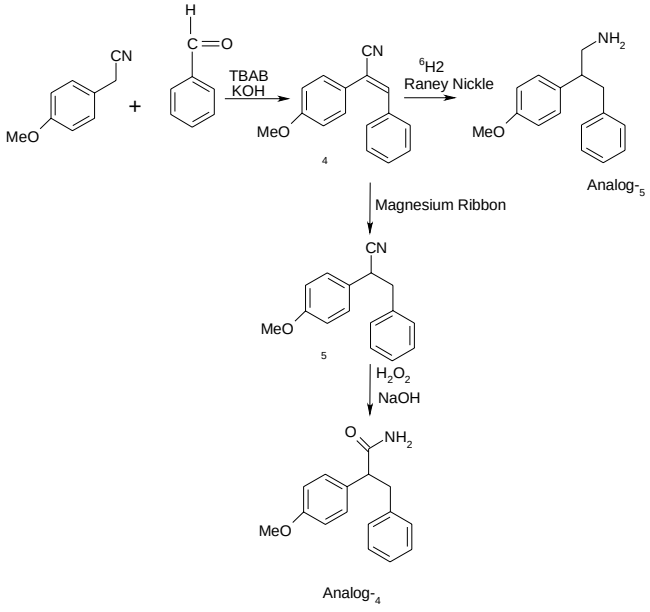
Compounds **2** (0.02M) was dissolved in 50 mL of Toluene. Na_2CO_3 (0.03M) and diphenyl methylene chloride (0.024M) was added and the reaction mixture was refluxed

for 3 hours, cooled, added to water and extracted with ethyl acetate and ethyl acetate layer was distilled to collect the sample as oily liquid, **Analog-3,** yield 93.6%. **IR**; 3400 (OH), 1248, 1033 (C-O-C) cm^{-1}; **^{1}H NMR** ($CDCl_3$); δ6.82,7.15 and 7.32 (m,14H,ArH), 5.85 (s,1H,CH_2NH), 4.76 (s,1H,NHCH), 3.82 (s,3H,OCH_3), 3.20 (m,2H,$CHCH_2$), 3.00 (m,1H,$CHCH_2$), 1.0-1.72 (m,10H,Cyclohexyl H).

Scheme – II: Synthesis of- 2-(4-methoxy-phenyl)-3-phenyl-acrylonitrile. (4)

Potassium hydroxide (0.071M) and TBAB (0.007M) was dissolved in 40 mL of methanol, cooled to 0-5ºC, over ice bath. The mixture of p-methoxy phenyl acetonitrile (0.1M) and benzaldehyde (0.1M) was added drop wise to the above solution over a period of half an hour. Then the reaction mixture was vigorously stirred at 0-5ºC, for 2 hours. The light yellow coloured solid obtained was filtered and washed several times with water, and recrystallised from methanol to get the product (**4),** m.p. 80 – 82º C, yield 89.28%.

Scheme-II



Synthesis of 2-(4-methoxy –phenyl)-3-phenyl-propionitrile.(5)

Compound **4** (0.04 M) is dissolved in 40 mL of methanol. To above solution add 7 g of dry magnesium ribbon slowly the reaction mixture is stirred for four hour, the methanol is distilled to collect the residue is dissolved in water and acidified with ethyl acetate. The ethyl acetate layer is separated and distilled to collect the solid sample and dried **5,** m.p.60-62ºC, yield 90.07%.

Synthesis of 2-(4-methoxy –phenyl)-3- phenyl-propionamide. Analog- 4

Compound **5** (0.02 M) is dissolved in 50 mL of 95% ethyl alcohol .to the above solution add 6 ml of 30% of hydrogen peroxide solution and 1.3 mL of 6N sodium hydroxide .the reaction mixture is stirred for 4 hour at 45-50ºC after reaction is complete the reaction mixture is neutralized with acetic acid then extracted with ethyl acetate layer is separated and distilled to collect solid sample **analog 4**.**IR**; 3400-3200(NH_2),1247,1033 (C-O–C),1650 (C=O) cm^{-1} ; **^{1}H NMR** ($CDCl_3$) ; δ6.96-7.42 (m,9H,ArH), 6.73 (s,2H,NH_2), 3.8(s,3H,OCH_3), 3.4 (m, 2H, $CHCH_2$), 2.8 (m,1H,$CHCH_2$).

Synthesis of 2-(4-Methoxy-phenyl)-3-phenyl-propylamine. Analog-5.

Compound **(4)** (0.043M) was dissolved in 300 ml of methanol in hydrogenator chamber, to this triethylamine (0.013M) was added and the reaction mixture was hydrogenated over Raney Nickel at 50-60ºC for 4 - 5 hours, then the catalyst was filtered, the filtrate was distilled to collect the oil, to this 50 ml of water was added and the solution was made pH-4 with acetic acid. Then aqueous layer was washed with toluene 2-3 times. The pH of the aqueous layer was adjusted to 8 with solid Na_2CO_3, the product formed was extracted with ethyl acetate. The ethyl acetate was distilled to get the product as oily liquid, **Analog-5**. **IR**; 3400-3300 (NH_2), 1247, 1033 (C-O-C) cm^{-1}; **^{1}H NMR** ($CDCl_3$); δ6.82-7.56 (m, 9H,ArH), 3.75(s, 3H,OCH_3), 2.9(s, 2H,CH_2CHCH_2), 2.75(m, 1H,$CHCH_2$), 2.5(s (b), 2H,CH_2CHCH_2).

Antidepressant activity

Antidepressant activity was screened by using FST. [5,6,7] Standard Venlafaxine and 1% w/v tragacanth solution was chosen as reference standard and control respectively. All the analogs including synthesized venlafaxine were given orally as 1% w/v tragacanth solution in the dose of 16 and 32mg/kg body weight, 1 hour prior the test session. Mouse was judged to be immobile, when it is ceased struggling and remained floating in the up right position, and made only small movements to keep its head above water. The time of immobility in seconds were recorded during last five minutes of testing period. Significant activity was obtained from all the analogs against control treated animals. Analog-1 and 3 at 16 mg/kg body weight were having antidepressant activity comparable to standard venlafaxine. Analog-1 exhibited better antidepressant activity at 32mg/kg body weight than standard venlafaxine. Statistical analysis was done using unpaired student T-test; the results are tabulated in table- I-III.

Table-I:
Comparison of control with other groups at 16 mg/kg body weight.

Groups	Compound	MAT[a] ± SEM[b]	P value	t-test
1	Control	207.1667 ± 4.6325	——	——
2	Standard	57.5000 ± 4.8058	P<0.0001	***
3	Synthesized [c]	61.6667 ± 2.4817	P<0.0001	***
4	Analog 1	41.6667 ± 8.3537	P<0.0001	***
5	Analog 2	93.3333 ± 4.4870	P<0.0001	***
6	Analog 3	51.6667 ± 7.2330	P<0.0001	***
7	Analog 4	83.6667 ± 7.2469	P<0.0001	***
8	Analog 5	109.0000 ± 9.1127	P<0.0001	***

[a]Mean antidepressant activity. [b]Standard error mean. [c]Synthesized Venlafaxine. *** = P < 0.0001.

Table-II:
Comparison of standard Venlafaxine with synthesized Venlafaxine and analogs at 16 mg/kg body weight.

Groups	Compounds	MAT[a] ± SEM[b]	P value	t-test
1	Standard	57.5000 ± 4.8058	——	——
2	Synthesized	61.6667 ± 2.4817	0.1301	ns
3	Analog 1	41.6667 ± 8.3537	0.1301	ns
4	Analog 2	93.3333 ± 4.4870	0.0003	***
5	Analog 3	51.6667 ± 7.2330	0.5154	ns
6	Analog 4	83.6667 ± 7.2469	0.0129	*
7	Analog 5	109.0000 ± 9.1127	0.0005	***

[a]Mean antidepressant activity. [b]Standard error mean. [c]Synthesized Venlafaxine. * = P < 0.01. *** = P < 0.0001. ns = not significant.

Table-III:
Comparison of standard Venlafaxine with synthesized Venlafaxine and analogs at 32 mg/kg body weight.

Group	Compounds	MAT[a] ± SEM[b]	P value	t-test
1	Standard	57.5000 ± 4.8058	——	——
2	Synthesized	61.6667 ± 2.4817	0.4572	ns
3	Analog 1	33.5000 ± 7.8823	0.026	*
4	Analog 2	69.6667 ± 10.2464	0.3058	ns
5	Analog 3	71.8333 ± 11.8283	0.286	ns
6	Analog 4	59.5000 ± 11.6979	0.877	ns
7	Analog 5	70.6667 ± 12.9567	0.3614	ns

[a]Mean antidepressant activity. [b]Standard error mean. [c]Synthesized Venlafaxine. * = P < 0.01. ns = not significant.

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Synthesis, Characterization and Antimicrobial Activity of some Quinoxaline derivatives and their Metal Complexes

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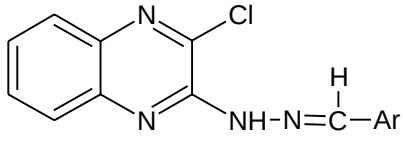
ABSTRACT

The complexes of oxoV^{IV}, Cr^{III}, Fe^{III}, Mn^{II}, Co^{II}, Ni^{II}, Cu^{II}, Zn^{II} and Pd^{II} complexes of hydrazones derived from the condensation reaction of 3-chloro-2-hydrazinequinoxaline with 2-hydroxybenzaldehyde, 2-hydroxy-3-methoxy-benzaldehyde and 2-hydroxy-1-naphthaldehyde have been prepared and characterized. All the three ligands function as mononegative, bidentate/tridentate ones coordinating with the metal ions through phenolic oxygen and azomethine nitrogen/ring C=N nitrogen. The geometry and the bonding characteristics associated with the complexes have been deduced from the relevant spectral data. Further, some selected compounds have been screened for their antimicrobial activity and the results are presented.

KEYWORDS: Quinoxaline hydrazone complexes, synthesis, characterization, antimicrobial activity.

Introduction

Quinoxalines are a class of fused six-membered nitrogen heterocyclics containing two nitrogens in mutually para disposition. These compounds have a wide range of applications in pharmacology, bacteriology and mycology [1-6]. These compounds have potent donor groups and despite this, the studies directed towards exploring the ligational behaviour of these compounds are limited. For this reason, we report, herein, the synthesis and characterization of oxoV^{IV}, Cr^{III}, Fe^{III}, Mn^{II}, Co^{II}, Ni^{II}, Cu^{II}, Zn^{II} and Pd^{II} complexes of some quinoxaline hydrazone derivatives namely 2-hydroxybenzaldehyde-1-(3-chloro-2-quinoxalinyl) hydrazone [HBCQOH], 2-hydroxy-3 methoxybenzaldehyde-1-(3-chloro-2quinoxalinyl) hydrazone [HMBCQH] and 2-hydroxy-1-naphthaldehyde-1-(3-chloro-2-quinoxalinyl)hydrazone [HNCQOH] (Fig.1), followed by screening of selected compounds for their antimicrobial activity.



Ar = 2-OHC_6H_4, 2-OH-3-$OMeC_6H_3$, 2-$OHC_{10}H_6$

Fig. 1

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Material and Methods

All the chemicals used were of A.R. or B.D.H. grade. 3-chloro-2-hydrazinoquinoxaline (i) was prepared as reported earlier [7]. The ligands HBCQOH, HMBCQH and HNCQOH were synthesized by stirring equimolar quantities of (i) and the respective aldehydes in DMF, for 2 hrs. at room temperature. OxoV^{IV} complexes of the ligands were prepared taking vanadyl sulphate, Mn^{II}, Co^{II}, Ni^{II} Cu^{II} and Zn^{II} complexes taking respective metal acetates and Cr^{III}, Fe^{III} and Pd^{II} complexes using respective metal chlorides. In the preparation of the metal complexes, the metal and the ligand were combined in 1:2 mole ratio using required quantities of methanol or water for the metal salts and methanol for the ligands.The contents were refluxed on a water bath for 2-3 hrs, the solid that separated was filtered, washed with water, hot methanol and ether and dried in air.

The elemental analyses were carried out by Carlo Erba 1108 elemntal analyzer at CDRI, Lucknow. Conductance measurements were made in DMF at 10^{-3} M concentration on a Digisun digital conductivity meter DI 909 model. Gouy balance calibrated with $Hg[Co(SCN)_4]$ was used to measure the magnetic susceptibility of the metal complexes at room temperature. The infrared spectra of the ligands and the metal complexes were recorded in KBR pellets in the range 4000 – 400cm^{-1} on Perkin Elmer-BX spectrophotometer at Central Instrumentation Center, Kakatiya University. The electronic spectra of the metal complexes in DMF were recorded on ELICO SL-159 UV-Vis spectrophotometer. The JEOL FE1X ESR spectrometer

operating in the frequency range 8.8 - 9.6GHz available with Department of Physics, Tirupati was employed in recording the ESR spectra of Cu(II) and oxoV(IV) complexes in DMF solution at LNT. The ^{1}H-NMR spectra of the ligands and their Zn(II) complexes were recorded in DMSO-d_6 solution employing Bruker avance 300 MHz spectrometer. The ligand HBCQOH and its complexes were assayed at the concentration 0.1mg/ml in acetone for their activity against the fungus: *Fusarium oxysporum* by glass humid chamber technique [8] and against the bacteria: *Staphylococcus aureus* (gram +ve) and *Escherichia coli* (gram –ve) by paper disc method [9].

Results and Discussion

All the metal complexes (Table 1) are coloured, stable at room temperature and are non - hygroscopic. The metal complexes decompose, upon heating, without melting. The ligands and their metal complexes are insoluble in water, slightly or very slightly soluble in methanol and acetone and fairly soluble in dimethyl formamide.

The Cr(III) and Fe(III) complexes record conductance values in the range 68-78 ohm^{-1} cm^2 mol^{-1} in DMF indicating that they are 1:1 electrolytes. The VO(IV), Mn(II), Co(II), Ni(II), Cu(II), Zn(II) and Pd(II) complexes are, on the contrary, shown to be non-electrolytic with only residual conductance values.

Thermal studies of the selected metal complexes namely VO(IV), Cr(III), Fe(III), Mn(II), Zn(II) and Pd(II) complexes of HBCQOH indicate that they are thermally stable to different temperatures. They lose weight on heating almost in a continuous manner attaining constancy in weight at different temperatures, for example, VO(IV) complex above 600^0, Cr(III) complex above 700^0, Mn(II) complex above 900^0, Fe(III) and Zn(II) complexes above 660^0 and Pd(II) complex above 680^0C. The per cent weight loss as computed from the thermograms of the complexes suggests that the final product of decomposition in all the cases, corresponds, within the permissible experimental error, to the respective metal oxide.

Table 1:
Analytical & physical data of metal complexes

Metal complex	Colour	Metal % Found (Cal)	Molar cond. Ω^{-1} cm^2 mol^{-1}	μ_{eff} B.M.
$VO(HBCQOH\text{-}H)_2$	Brown	7.38 (7.69)	16	1.71
$Cr(HBCQOH\text{-}H)_2Cl$	Yellowish green	7.55 (7.61)	72	3.81
$Fe(HBCQOH\text{-}H)_2Cl$	Dark brown	8.02 (8.13)	75	5.72
$Mn(HBCQOH\text{-}H)_2$	Dark red	8.29 (8.45)	12	5.69
$Co(HBCQOH\text{-}H)_2$	Orange red	8.92 (9.01)	14	4.68
$Ni(HBCQOH\text{-}H)_2$	Orange red	8.75 (8.97)	10	2.98
$Cu(HBCQOH\text{-}H)_2$	Dark brown	9.45 (9.64)	12	1.84
$Zn(HBCQOH\text{-}H)_2$	Yellow	9.73 (9.89)	13	—
$Pd(HBCQOH\text{-}H)_2$	Orange	15.10 (15.16)	10	—
$VO(HMBCQH\text{-}H)_2$	Dark brown	6.93 (7.05)	12	1.71
$Cr(HMBCQH\text{-}H)_2Cl$	Brown	6.82 (7.00)	68	3.80
$Fe(HMBCQH\text{-}H)_2Cl$	Dark brown	7.50 (7.48)	72	5.70
$Mn(HMBCQH\text{-}H)_2$	Dark red	7.55 (7.73)	13	5.72
$Co(HMBCQH\text{-}H)_2$	Orange	8.13 (8.25)	16	4.80
$Ni(HMBCQH\text{-}H)_2$	Brown	8.20 (8.22)	14	3.25
$Cu(HMBCQH\text{-}H)_2$	Dark brown	8.65 (8.84)	12	1.83
$Zn(HMBCQH\text{-}H)_2$	Yellow	8.89 (9.07)	12	—
$Pd(HMBCQH\text{-}H)_2$	Yellow	13.65 (13.97)	13	—
$VO(HNCQOH\text{-}H)_2$	Dark brown	6.52 (6.68)	14	1.70
$Cr(HNCQOH\text{-}H)_2Cl$	Brown	6.49 (6.64)	73	3.83
$Fe(HNCQOH\text{-}H)_2Cl$	Dark brown	7.02 (7.10)	78	5.74
$Mn(HNCQOH\text{-}H)_2$	Dark red	7.12 (7.32)	12	5.72
$Co(HNCQOH\text{-}H)_2$	Orange	7.69 (7.81)	15	4.84
$Ni(HNCQOH\text{-}H)_2$	Brown	7.65 (7.78)	16	3.24
$Cu(HNCQOH\text{-}H)_2$	Dark brown	8.15 (8.37)	12	1.81
$Zn(HNCQOH\text{-}H)_2$	Yellow	8.32 (8.59)	12	—
$Pd(HNCQOH\text{-}H)_2$	Yellow	13.02 (13.27)	14	—

The magnetic studies made on the complexes of all the ligands indicate that VO(IV), Cr(III), Fe(III), Mn(II), Co(II), Ni(II) and Cu(II) complexes are paramagnetic corresponding to one, three, five, five, three, two and one unpaired electrons respectively and that Zn(II) and Pd(II) complexes are diamagnetic.

IR Spectra

All the ligands show, in their spectra, a medium intensity band in the region 3200-3330 cm^{-1} that has been assigned to νO-H. This band disappears in the spectra of their complexes indicating that deprotonation of the group has taken place. A small or medium intensity band around 1230cm^{-1} in the ligands assignable to νC-O is seen to have undergone a positive shift by 30-50cm^{-1} in the complexes suggesting coordination through phenolic oxygen [10]. The positive shift observed may be attributed to the drift of electron density from oxygen to the metal ion resulting in greater ionic character of the C-O bond and a consequent increase in its vibration frequency [11]. The ligands record a somewhat broad, medium intensity band around 3430cm^{-1} attributable to free νN-H [12]. This band remains either unshifted or higher shifted in the complexes indicating nonparticipation of nitrogen of this group in coordination. Further, the ligands reveal bands around 1620cm^{-1} due to free νC=N and around 1580cm^{-1} due to ring νC=N. While the band due to free νC=N has got lower shifted by 10-20cm^{-1} in all the complexes, the band due to ring νC=N is lower shifted by 20-30cm^{-1} in the Cr(III), Mn(II), Fe(III), Co(II), Ni(II) and Cu(II) complexes and remains unshifted in VO(IV), Zn(II) and Pd(II) complexes. This suggests that the ligands act as mononegative, bidentate ones bonding through phenolic oxygen and nitrogen of free C=N group in VO(IV), Zn(II) and Pd(II) complexes and as mononegative, tridentate ones coordinating additionally through nitrogen of ring C=N in Cr(III), Mn(II), Fe(III), Co(II), Ni(II) and Cu(II) complexes [13-15].

An intense band that appears around 750cm^{-1} in all the ligands and their metal complexes has been assigned to νC-Cl [16] and fairly intense band that figures around 953cm^{-1} in all the VO(IV) complexes has been attributed to νV=O [17].

The coordination through phenolic oxygen and azomethine nitrogen(s) is further substantiated by the appearance, in all the complexes, of non-ligand bands in the far infrared region around 500 and 400cm^{-1} assignable repectively to νM-O and νM-N vibrations [18,19].

^{1}H-NMR Spectra:

The ^{1}H-NMR spectra of the ligands and their Zn(II) complexes are recorded. A comparison of the spectra of the ligands and the Zn(II) complexes clearly indicates deprotonation of phenolic OH during complexation. This suggests participation of phenolic OH group in coordination.

Electronic Spectra:

The electronic spectral frequencies observed for the complexes along with ligand field paramenters for the Co(II) and Ni(II) complexes are given in Table 2. These frequencies may be assigned, in the increasing order, to the transitions as detailed below [20].

Oxo V(IV) complexes :

$^2B_2 \rightarrow {}^2E_2$, $^2B_2 \rightarrow {}^2B_1$ and $^2B_2 \rightarrow {}^2A_1$

Cr(III) complexes:

$^4A_{2g} \rightarrow {}^4T_{2g}$, $^4A_{2g} \rightarrow {}^4T_{1g}(F)$ and $^4A_{2g} \rightarrow {}^4T_{1g}(P)$.

Fe(III) complexes:

$^6A_{1g} \rightarrow {}^4T_{1g}(G)$, $^6A_{1g} \rightarrow {}^4T_{2g}(G)$ and $^6A_{1g} \rightarrow {}^4E_g(G)$

Co(II) complexes:

$^4T_{1g}(F) \rightarrow {}^4T_{2g}(F)$, $^4T_{1g}(F) \rightarrow {}^4A_{2g}(F)$ and $^4T_{1g}(F) \rightarrow {}^4T_{1g}(P)$

Ni(II) complexes:

$^3A_{2g}(F) \rightarrow {}^3T_{2g}(F)$, $^3A_{2g}(F) \rightarrow {}^3T_{1g}(F)$ and $^3A_{2g}(F) \rightarrow {}^3T_{1g}(P)$

Cu(II) complexes:

$^2B_{1g} \rightarrow {}^2B_{2g}$ and $^2B_{1g} \rightarrow {}^2E_g$

Pd(II) complexes:

$^1A_{1g} \rightarrow {}^1A_{2g}$, $^1A_{1g} \rightarrow {}^1B_{1g}$ and $^1A_{1g} \rightarrow {}^1E_g$

Based on these transitions and the other data observed, square pyramidal geometry for the oxoV(IV) complexes, octahedral geometry for the Fe(III), Cr(III), Co(II), and Ni(II) complexes, tetragonal geometry for the Cu(II) complexes and square planar geometry for the Pd(II) complexes have been proposed [21,22] Further, the ligand field parameters 10 DQ, B and β obtained for the Co(II) and Ni(II) complexes indicate that the metal-ligand bond in them is covalent in nature [23].

ESR Spectra:

The ESR spectral parameters calculated for the oxoV(IV) and Cu(II) complexes using appropriate methods and equations [24] are presented in Table-3. The spectra of all the oxoV(IV) complexes are well resolved with eight parallel and eight perpendicular components due to hyperfine coupling with vanadium nucleus I = 7/2. The spectra of Cu(II) complexes are of anisotropic nature in that each of them has two peak envelopes, one of small intensity towards low field and other of large intensity towards high field. The small intensity envelope towards low field has been resolved into two to four peaks due to hyperfine interaction with copper nucleus (I = 3/2) while the large intensity peak towards high field remains unresolved. The g and A values observed for the oxoV(IV) complexes are in agreement with those generally observed for a vanadyl complex with a square pyramidal geometry [25]. For all the complexes, $g_{\parallel} < g\perp < g_e$ (where g_e is free

Table-2:
Electronic spectral data of the metal complexes of HBCQOH, HMBCQH,and HNCQOH

Metal complex	Frequency (cm^{-1})			10DQ cm^{-1}	B cm^{-1}	β
VO-HBCQOH	14480	16660	25500	—	—	—
Cr-HBCQOH	16500	23000	32000	—	—	—
Fe-HBCQOH	12500	15600	20000	—	—	—
Mn-HBCQOH	13120	19180	24610	—	—	—
Co-HBCQOH	9300(ν_1)	16220(ν_2)	22980(ν_3)	6918	753	0.775
Ni-HBCQOH	9530(ν_1)	14200(ν_2)	24300(ν_3)	9530	661	0.642
Cu-HBCQOH	15250	20350	—	—	—	—
Pd-HBCQOH	14970(ν_1)	18650(ν_2)	23360(ν_3)	—	—	—
VO-HMBCQH	14450	16680	25470	—	—	—
Cr-HMBCQH	16480	23750	31950	—	—	—
Fe-HMBCQH	12410	15550	20450	—	—	—
Mn-HMBCQH	13110	19190	25000	—	—	—
Co-HMBCQH	9320(ν_1)	16630(ν_2)	22800(ν_3)	7312	765	0.78
Ni-HMBCQH	9270(ν_1)	14010(ν_2)	23950(ν_3)	9270	677	0.65
Cu-HMBCQH	15230	20370	—	—	—	—
Pd-HMBCQH	14650(ν_1)	17830(ν_2)	22680(ν_3)	—	—	—
VO-HNCQOH	14480	16570	25460	—	—	—
Cr-HNCQOH	16490	23000	31960	—	—	—
Fe-HNCQOH	12440	14210	20390	—	—	—
Mn-HNCQOH	13090	19110	24690	—	—	—
Co-HNCQOH	9370(ν_1)	16800(ν_2)	22180	7426	725	0.747
Ni-HNCQOH	9430(ν_1)	14090	23810	9430	641	0.622
Cu-HNCQOH	15650	19900	—	—	—	—
Pd-HNCQOH	14700(ν_1)	17890(ν_2)	22680(ν_3)	—	—	—

Table-3:
ESR parameters of VO(IV) and Cu(II) complexes

Complex	$g_{\|\|}^{*}$	$g\perp^{*}$	g_{av}^{**}	α^2	β^2	γ^2
VO-HBCQOH	1.95	1.99	1.98	—-	—-	—-
VO-HMBCQH	1.94	2.00	1.98	—-	—-	—-
VO-HNCQOH	1.94	1.98	1.97	—-	—-	—-
Cu-HBCQOH	2.24	2.05	2.11	0.55	0.93	0.80
Cu-HMBCQH	2.25	2.05	2.11	0.58	0.92	0.78
Cu-HNCQOH	2.24	2.05	2.12	0.59	0.86	0.75

electron value) which indicates that the unpaired electron is in the d_{xy} orbital with ^2B as the ground state [26]. In the Cu(II) complexes, it is found that $g_{\|} > g\perp > 2$ indicating that the unpaired electron lies in d_{x2}-d_{y2} orbital with $^2B_{1g}$ as the ground state [27]. The α^2, β^2 and γ^2 values obtained for the present Cu(II) complexes are in the ranges 0.55-0.59, 0.86-0.93 and 0.75-0.80 suggesting appreciable / weak/ moderate in-plane σ-bonding, in-plane π-bonding and out-of-plane π-bonding respectively [28].

Antimicrobial activity:

The activity profiles of the ligand HBCQOH and its metal complexes screened against the microorganisms are varying in that some of the compounds are active either significantly or marginally while others are not. Further, it may be noted that the compounds are more antifungal than antibacterial.

Antifungal activity: The antifungal results indicate that the metal complexes do, in general, well in that many of them are either more active than or as active as their ligand. While the activity of Cr and Mn complexes is on par with that of the ligand, the other metal complexes are more active amongst which the Zn complexes are endowed with highest level of activity.

Antibacterial activity: The results on the antibacterial activity of the compounds screened indicate that the ligand HBCQOH does not exert activity on the microorganisms, as are its Mn, Fe and Co complexes. While the Cr, Cu and Zn complexes are active, to a different level, of course, against both the bacteria, the others are so either with gram +ve or with gram –ve bacterium. The Hg complex, of all, has been found most active.

The activity exerted by the compounds may be attributed, in general terms, to their preferential penetration of the microorganism cell wall and interfering, therein, with the normal functions of the microorganism.

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Synthesis and Antimicrobial screening of some new Acid chloride derivatives of 2-amino-N- (3-chlorophenyl)-4,5,6,7-tetrahydrobenzo [b] thiophen-3- Carboxamide

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ABSTRACT

2-amino-N- (3-chlorophenyl)-4,5,6,7-tetrahydrobenzo [b] thiophen-3-Carboxamide were synthesized by Gewald reaction. Later the compound Ia is treated with ten different acid chlorides to yield a novel series of thiophenes (II-XIa). IR, NMR, Mass spectral data and elemental analysis characterized the synthesized compounds. All the synthesized compounds were screened for their Antimicrobial activity. Some of the products exhibited interesting activity with known standard drug at same concentration.

KEY WORDS: Synthesis, Thiophenes, Acid chloride derivatives, and Antibacterial activity.

Introduction

A variety of thiophene compounds such as Sertaconazole, Tioconazole, benzothiophene-3-carboxamide esters and substituted benzo [b] thiophenes [1-6] have been examined as Antifungal agents, Similarly thiophenes containing β-lactam antibiotics like ticarcillin, ticlopidine, cephaloridine, cephalothin & cefoxitin have shown good antibacterial activity, Chlorine containing β-lactam antibiotics like cloxacillin, dicloxacillin have shown good antibacterial activity. Clotrimazole, miconazole, fenticonazole, itraconazole, and ketaconazole containing chlorine substitution possess good Antifungal activity. Hence a novel series of thiophene compounds with chlorine substitution have been synthesized and screened for antibacterial and antifungal activity [7].

Antimicrobial Activity:

All the synthesized compounds (IIa-XIa) were screened for antibacterial by agar diffusion method [8] and antifungal activity (by Czapek-Dox agar media [8] (P^H 7.3) at a concentration 50 µg using DMF as solvent .The zone of inhibition was measured in mm and the average of 3 readings were taken, and presented in Table-2. The activity was compared with Ampicillin and Miconazole nitrate at the same concentration.

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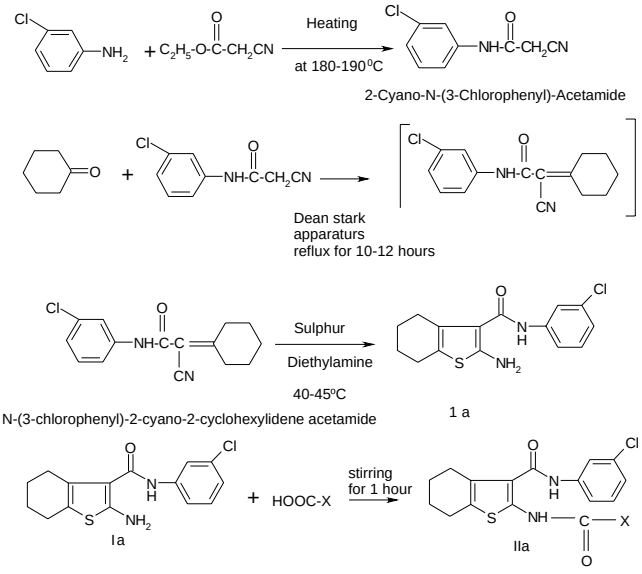
Materials and Methods

Scheme



Experimental

Melting points are uncorrected. The purity of the synthesized compounds was checked by thin layer chromatography. Characterization of synthesized compounds was done by spectral studies. The infrared spectra were determined on Shimadzu

435-IR spectrophotometer using potassium bromide discs.^{1}H NMR spectra were recorded on Bruker AMX 400 using TMS as internal standard. The chemical shift values are in delta (ppm). Physical data and Antimicrobial activities of newly synthesized compounds are recorded in Table-1 & Table-2.

General method for the synthesis of the New Acid chlorides

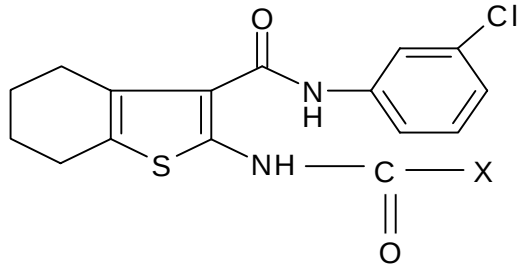
A mixture of substituted aromatic acid (0.005mol) and phosphorous oxychloride (0.015mol) was refluxed for 4hours;excess phosphorous oxychloride was distilled off under vacuum [9,10] and immediately processed for next step.

Synthesis of Acid chloride of 2-amino-N- (3-chlorophenyl)-4,5,6,7-tetrahydrobenzo [b] thiophen-3-Carboxamide:- (Ia)

The starting compound 2-amino-N- (3-chlorophenyl)-4,5,6,7-tetrahydrobenzo [b] thiophen-3- Carboxamide were synthesized by already reported procedure [11]. Later to the compound 2-amino-N- (3-chlorophenyl)-4,5,6,7-tetrahydrobenzo [b] thiophen-3- Carboxamide [12-15] (1.46g; 0.005mol) in dry pyridine was added, the freshly prepared p-chloro benzoyl chloride drop wise with stirring for 1hr.The reaction mixture was poured into crushed ice and the solid obtained was washed with ice cold water. Recrystallized from DMF/water mixture. Yield 72 % m.p135^{o}C.The other compounds reported in table were prepared in the same manner.

Results

Table-1
Physical data of Synthesized compounds



IIa

Compound	X	M.P	% Yield	Molecular Formula	R_f value	TLC Solvent system
IIa	2-bromophenyl	110^{o}C	49	$C_{22}H_{18}BrClN_2O_2S$	0.866	Benzene:chloroform (7:3)
IIIa	4-chlorophenyl	135^{o}C	72	$C_{22}H_{18}ClN_2O_2$ S	0.845	Benzene:chloroform (5:5)
IVa	2-hydroxyphenyl	94^{o}C	43	$C_{21}H_{16}Cl_2N_2O_2$ S	0.736	Benzene:chloroform (8:2)
Va	2-amino-5-bromophenyl	155^{o}C	47	$C_{22}H_{19}BrClN_3O_2S$	0.636	Benzene:chloroform (7:3)
VIa	2-aminophenyl	110^{o}C	52	$C_{22}H_{20}ClN_3O_2S$	0.53	Benzene:chloroform (7:3)
VIIa	2-chlorophenyl	110^{o}C	67	$C_{22}H_{18}Cl_2N_2O_2S$	0.754	Benzene:chloroform (7:3)
VIIIa	2-hydroxy-3,5-dinitrophenyl	145^{o}C	69	$C_{22}H_{17}ClN_4O_7$ S	0.70	Petroleum ether: Ethylacetate(8:2)
IXa	4-aminophenyl	127^{o}C	66	$C_{22}H_{20}ClN_30_2S$	0.566	Benzene:chloroform (7:3)
Xa	Acetyl	124^{o}C	63	$C_{21}H_{16}Cl_2N_2O_2S$	0.39	Benzene:chloroform (7:3)
XIa	3-phenylacryl amido	132^{o}C	55	$C_{24}H_{21}ClN_20_2S$	0.843	Benzene:chloroform (7:3)

Table-2
Anti microbial activity data

Compound No.	Antibacterial activity Zone of Inhibition in mm			Antifungal activity Zone of inhibition in mm		
	E.Coli	Klebsiella	S.Aureus	B.Subtilis	A. Niger	Cladosporium
IIa	2	2	2	2	NA	2
IIIa	3	2	1	1	2	4
IVa	NA	2	1	NA	NA	NA
Va	4	2	3	2	NA	NA
VIa	2	1	NA	NA	NA	NA
VIIa	NA	4	NA	NA	2	4
VIIIa	10	8	4	4	7	5
IXa	NA	NA	NA	NA	NA	NA
Xa	NA	NA	NA	NA	NA	NA
XIa	7	2	4	1	2	1
Ampicillin	19	20	14	13	–	–
Miconazole Nitrate	–	–	–	–	20	16

(NA) – No activity
DMF – Di methyl Formamide
Control – DMF
Dose concentration in mcg – 0.1ml

Discussion

The formation of the starting compound 2-amino-N-(3-chlorophenyl)-4,5,6,7-tetrahydrobenzo [b] thiophen-3-Carboxamide (Ia) from 2-Cyano-N-(3-chlorophenyl)-Acetamide has been clearly indicated by the characteristic IR spectra, which show absorption band at 3474.5cm^{-1} (-NH_2-) and 3354 cm^{-1} (-NH-) arising from the asymmetric and symmetric stretching vibrations, further the presence of a sharp band at 2900-2700 cm^{-1} is due to (-S-CH-) group.

Since amides have a very strong tendency to self-associate by hydrogen bonding the band positions have been shifted to lower frequency region. It is observed the spectra show characteristic sharp band at 1637.5 cm^{-1} (C=O), which is due to stretching vibration of carbonyl group. The IR spectra of compound Ia showed distinct peaks at 3474.5cm^{-1}(NH_2), 3354 cm^{-1} (NH), 1637.5 cm^{-1}(C=O), 2940.3 cm-1 (Ar-H), 1477.4 cm^{-1}, 1230.5 cm^{-1} (CH_2), 1568.0 cm^{-1}, 1519 cm^{-1}, (Ar-C=C), 773.4 $cm^{-1,}$ 677 cm^{-1} (C-Cl) .The presence of nitrogen and sulphur was confirmed by Lassaigne's test. All the above observations with difference in the TLC spots, confirm the formation of 2-amino-N- (3-chlorophenyl)-4,5,6,7-tetrahydrobenzo [b] thiophen-3- Carboxamide (Ia).

The next step was the synthesis of 2- (4-chloro benzamido)-N- (3-chlorophenyl)-4,5,6,7-tetrahydrobenzo [b]- thiophen-3-carboxamide (III-a) carried out by the reaction of 2-amino-N- (3-chlorophenyl)-5,6-dihydrocyclopenta [b] thiophen-3-carboxamide in pyridine (30ml) and freshly prepared 4-chloro -benzoyl chloride was added drop wise and stirred for 1 hour the reaction mixture was poured into crushed ice. The formation of the new compound was confirmed by the difference in the melting point and R_f values (TLC). The melting point of: 2- (4-chloro benzamido)-N- (3-chlorophenyl)-4,5,6,7-tetrahydrobenzo [b]- thiophen-3-carboxamide (Compound No-IIIa) was found to be 135°C while the melting point of 2-amino-N- (3-chlorophenyl)-4,5,6,7-tetrahydrobenzo [b] thiophen-3-carboxamide was found to be 118°C.

Further, the IR spectrum of the compound showed the peak at 3428.5cm^{-1} (NH), 2930.2 cm^{-1} (Ar-H), 1668.6 cm^{-1}(C=O), 1485 cm^{-1}, 1464.5 cm^{-1}, 1417.5 cm^{-1}, 1400 cm^{-1} (Ar-C=C), 877.7 cm^{-1} (C-Cl), 777.4 cm^{-1}, 665.5 cm^{-1} (C-Cl).

IR spectral values for synthesized compounds

Compound no- IIa: - 3439.5 Cm^{-1} (NH), 2949.5 Cm^{-1} (Ar-H), 1674.4 Cm^{-1}(C=O), 1589.5 Cm^{-1}, 1556.7 Cm^{-1}, 1400.5 Cm^{-1} (Ar-C=C) 777.4 Cm^{-1}, 681.0 Cm^{-1} (C-Cl) 534.3 Cm^{-1} (C-Br).

Compound no- IIIa: - 3428.5cm^{-1}(NH), 2930.2 cm^{-1} (Ar-H), 1668.6 cm^{-1}(C=O), 1485 cm^{-1}, 1464.5 cm^{-1}, 1417.5 cm^{-1}, 1400 cm^{-1}(Ar-C=C),877.7 cm^{-1}(C-Cl), 777.4 cm^{-1}, 665.5 cm^{-1} (C-Cl).

Compound no-IVa: - 2932 cm^{-1} (NH), 1744.5 cm^{-1}(C=0), 1587.3 cm^{-1}, 1515.9 cm^{-1}, 1479.3 cm^{-1} (Ar-C=C) 778.2 cm^{-1}, 683.7 cm^{-1} (C-Cl), 1356.2Cm^{-1}(OH).

Compound no- Va:- 3071.0 cm^{-1} (NH), 2930 cm^{-1} (NH_2), 1697.6, cm^{-1}(C=O),1591.5 cm^{-1},1541.3 Cm^{-1} 1479.6 cm^{-1}(Ar-C=C), 783.2 Cm^{-1},682.9 cm^{-1} (C-Cl),561.4 cm^{-1}(C-Br).

Compound no-VI a: - 3649.8 cm^{-1} (NH_2), 3447.2 Cm^{-1} (NH), 2932.2 cm^{-1} (Ar-H),1755.4 cm^{-1}(C=O),773.5 cm^{-1}681.0 cm^{-1}(C-Cl).

Compound no-VII a: - 3271.0 cm^{-1} (NH), 2932.6 cm^{-1} (Ar-H), 1671.2 cm^{-1}(C=O),1588.3 cm^{-1},1518.8 cm^{-1}1475.4 cm^{-1},1422.4 cm^{-1}(Ar-C=C),777.3 cm^{-1},614 cm^{-1}(C-Cl).

Compound no- VIII-a: - 3524 cm^{-1}(NH),2932.2 cm^{-1}(Ar-H), 1674 cm^{-1}(C=O),1508.5 cm^{-1},1385.1 cm^{-1}(Ar-NO_2), 777.1 cm^{-1},686.7 cm^{-1} (C-Cl)1419.8(C-OH).

Compound no-IX a: - 3711.5cm^{-1}, 3649.8 cm^{-1}(NH),1749.6 cm^{-1}, 775.5 cm^{-1}, 679.0 cm^{-1} (C-Cl).

Compound no-X a: - 3448.5 cm^{-1} (NH), 2932.6 cm^{-1} (Ar-H), 1404.1 cm^{-1}(CH_3),1677.0 cm^{-1} (C=O),1589 cm^{-1},1476 cm^{-1},1431 cm^{-1}(Ar-C=C), 775.3 cm^{-1},883 cm^{-1}(C-Cl)

Compound no –XI a: - 3649.8cm^{-1}(NH), 2980.2cm^{-1}(Ar-H), 3065.3cm^{-1}(C=C), C-Cl (775.3cm^{-1}, 681.0cm^{-1}).

^{1}H-NMR (300 MHz, $CDCl_3$, δ, ppm) COMPOUND NO- (IV-a)

1.8 (m, 4H, -CH2-), 2.7 (t, 3H,-CH2-), 3.9 (s, 1H,-OH-), 11.1 (s, 1H, -NH-), 6.9-8.0 (m, 8H,Arom)

The NMR spectrum of the compound indicated the formation of new compound.

The synthesized compounds were screened for antibacterial and antifungal activity. From the screening results it was observed that presence of nitro group at 3,5 position of phenyl ring has shown moderate Antifungal and antibacterial activity when compared to standard.

The presence of bromo group at 3,5 position of the phenyl group has shown appreciable antibacterial and low antifungal activity.

The presence of acrylic at para & ortho position of phenyl group made the compound to exhibit considerable antifungal activity and low antibacterial activity.

We provided a convenient synthetic method for the synthesis of new title compounds and the results of antibacterial and antifungal screening are encouraging. Further investigations with appropriate structural modifications of title compounds may result in therapeutically useful products.

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