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CONTENTS

VIEWS

Synthesis and biological activities of Schiff bases and Mannich bases derived from 4-aryl dihydropyrimidinones

MADHUSUDAN N. PUROHIT*, BHAVYA.K, GURUBASAVARAJ. V. PUJAR
Department of Pharmaceutical Chemistry, J.S.S. College of Pharmacy,
S.S.Nagar, Mysore, Karnataka- 570 015

ABSTRACT

4-aryl dihydropyrimidinone derivatives (Ia-Ib) were synthesized by classical Biginelli condensation. Dihydropyrimidinones (Ia-Ib) were regioselectively formylated by Villsmeier-Haack reaction to get 3-formyl derivatives (IIa-IIb), which were further converted into Schiff bases (IIIa-IIIj) by treating with different aryl amines in absolute ethanol. Mannich bases (IVa-IVj) were prepared by the reaction of Ia/Ib with paraformaldehyde and substituted secondary amines in absolute ethanol. All synthesized compounds were screened for their *in vivo* analgesic and anti-inflammatory activities. The *in vivo* analgesic activity was carried out in albino mice using Eddy's Hot Plate method, while *in vivo* anti-inflammatory activity was carried out in albino rats using Carragenan induced paw oedema method. Schiff bases III d, III h and Mannich bases IV c, IV h showed analgesic activity between 76-80% when administered at the dose of 50mg/kg body weight by oral route. Compounds III d and IV c showed 52% and 97% reduction in the oedema volume respectively at a dose of 50mg/kg body weight administered by oral route.

Key words: Biginelli Condensation; Dihydropyrimidinone; Villsmeier-Haack reaction; Schiff Base; Mannich Base.

Introduction

Dihydropyrimidinones (DHPM) closely resemble in structure to dihydropyridine like Nifedipine which is a potent calcium channel blocker and antihypertensive agent. Dihydropyrimidinones are considered as aza analogues of dihydropyridines [1]. These compounds are reported to possess wide spectrum of biological activities like calcium channel blocking, anti-hypertensive, antimicrobial, anti-convulsant, analgesic, anti-inflammatory and anti-retroviral [2]. Mannich and Schiff bases have been reported to possess various biological activities like antimicrobial, anti-convulsant, analgesic and anti-inflammatory [3]. Based on these observations it was thought to prepare Mannich and Schiff bases derived from 4-aryl-dihydropyrimidinone nucleus. The carbethoxy dihydropyrimidinone derivative that served as starting material for the present work was synthesized by Classical Biginelli condensation by suitable modification [4]. In this paper we report the synthesis of certain Mannich and Schiff bases derived from 4-aryl dihydropyrimidinone and their *in vivo* analgesic and anti-inflammatory activities.

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Materials and Methods

Melting points were determined in open capillary tube and are uncorrected. The purity of the compounds was checked by TLC using ethyl acetate and toluene (4:6) as solvent system and iodine vapors as visualizing agent. IR spectra were recorded on Schimadzu 8400-S by KBr pellet method. ^{1}H-NMR spectra were recorded on AMX 400 NMR Spectrophotometer at 400 MHz using deuteriated dimethyl sulfoxide (DMSO-d_6) as the solvent and Tetra methyl silane as internal standard. The scheme of the synthesis employed for the present study is depicted in **Fig 1**.

Preparation of 3-formyl-6-methyl-2-oxo-4-aryl-1, 4- dihydropyrimidine -5- carboxylic acid ethyl ester (IIa/IIb):

To a solution of I a/I b (0.02mole) in 20 ml of dry DMF, phosphorus oxy chloride (0.02mole) was added in ice bath. The resulting solution was heated at 70^0 C and kept at the same temperature with constant stirring for 1hour. After completion of the reaction (monitored by TLC), the solution was cooled and poured into 150ml of ice water. The precipitated product was filtered, dried and recrystallised from ethanol.

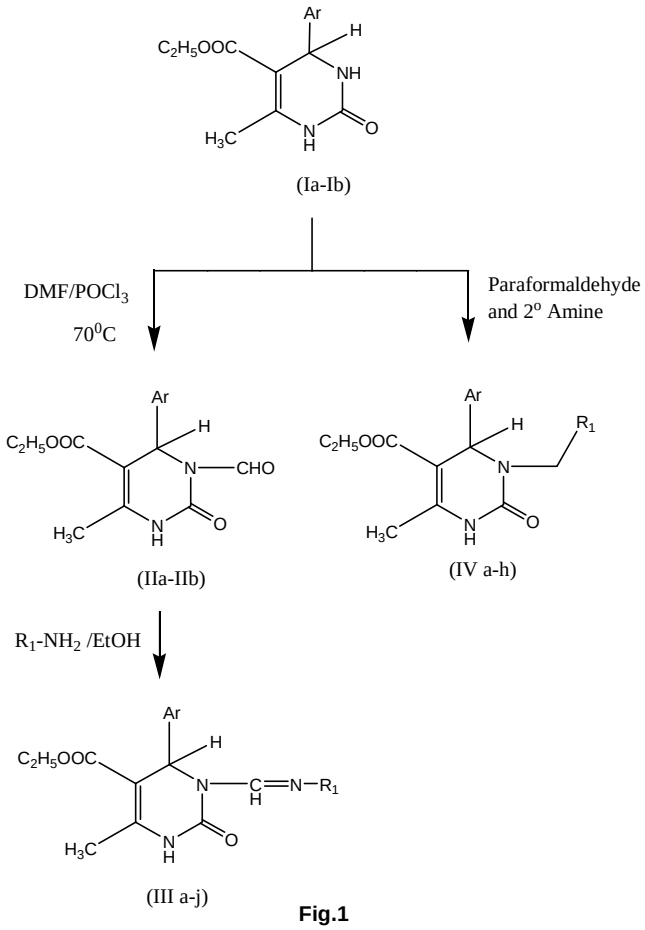

Fig.1

IIb: IR (KBr, cm^{-1}) C=O (Cyclic)-1645, C=O (Ester)-1705, N-H of DHPM-3250, N-H of DHPM-3122, aldehydic C-H 2972.

^{1}H-NMR (DMSO-d_6): ´ in ppm: 9.3 (1H, Aldehyde proton), 6.3 (1H, NH-DHPM), 6.8-7.3 (5H, Ar-H), 5.4 (1H, CH at C-4 of DHPM), 4.1(2H, OCH_2), 3.77 (3H, Ar-OCH_3), 2.4(3H, CH_3 at C-6 of DHPM), 1.1(3H, CH_3).

General procedure for the preparation of 3-aryliminomethyl-6-methyl-2-oxo-4-aryl-1,4-dihydropyrimidine-5-carboxylic acid ethyl ester (III a-j):

To a mixture of 0.01 mole of appropriate N-3 formyl DHPM (IIa/IIb) and aryl amines (0.01 mole) in 15ml of ethanol, 1ml of glacial acetic acid was added as a catalyst. The reaction mixture was refluxed for 20-24 hours. After completion of the reaction (monitored by TLC), the reaction mixture was poured into the crushed ice. The precipitated product was filtered and recrystallised from absolute alcohol.

III f: 3-phenyliminomethyl-6-methyl-2-oxo-4-(4-methoxy phenyl)-1,4-dihydropyrimidine-5-carboxylic acid ethyl ester.

IR (KBr, cm^{-1}) C=O (cyclic)-1645, C=O (ester) - 1708, CH=N- 2955, N-H of DHPM-3232, N-H of DHPM- 3105.

^{1}H-NMR (DMSO-d_6): ´ in ppm: 8.37 (1H, -CH=N), 6.8-7.26 (9H, Ar-H), 5.8 (1H, NH-DHPM), 5.3 (1H, CH at C-4 of DHPM), 4.1(2H, OCH_2), 3.7 (Ar-OCH_3), 2.3 (3H, CH_3 at C-6 of DHPM), 1.1(3H, CH_3).

Preparation of Mannich bases (IV a- h):

A mixture of 0.01mole of carbethoxy DHPM and 0.01mole of secondary amines in 5ml of ethanol, 0.033 mole of paraformaldehyde was added and the mixture was refluxed for 10-15 hours. After completion of the reaction (monitored by TLC), the reaction mixture was cooled and poured on to the crushed ice. The precipitated Mannich base was filtered, dried and recrystalised from ethanol.

IV h: 3-((piperazino) methyl)-6-methyl-2-oxo-4-(4-methoxy phenyl)-1, 4-dihydropyrimidine-5-carboxylic acid ethyl ester.

IR (KBr, cm^{-1}) C=O (cyclic) -1643, C=O (ester) - 1705, -CH_2- 2820, N-H of DHPM-3234, N-H of DHPM-3109.

^{1}H-NMR (DMSO-d_6): ´ in ppm: 6.8-7.2 (5H, Ar-H), 5.8(1H, NH-DHPM), 5.3 (1H, CH at C-4 of DHPM), 4.1(2H, OCH_2), 3.78 (3H, Ar-OCH_3), 3.69 (2H, -N-CH_2-N-), 2.45-2.6 (CH_2 of piperazine), 2.3(3H, CH_3 at C-6 of DHPM), 1.74 (1H, -NH of piperazine), 1.1(3H, CH_3).

Physical data of the synthesized compounds is summarized in **Table-1.**

Biological Activities

Analgesic activity

All the synthesized compounds were screened for their *in vivo* analgesic activity by Eddy's Hot plate method in albino mice using Diclofenac sodium as standard at a dose of 50mg/kg body weight [5]. Analgesic activity of compounds was measured in % analgesia.

Anti-inflammatory activity

All the synthesized compounds were screened for their *in vivo* anti-inflammatory activity by Carrageenan induced rat paw oedema method in albino rats using Diclofenac sodium as standard at a dose of 50mg/kg body weight [5]. Anti-inflammatory activity of compounds was measured in % oedema reduction.

Result and Discussion

The starting compounds 5-carbethoxy DHPM were synthesized by one step multi component Biginelli condensation. The spectral data are in agreement with proposed structure. The conversion of Ib to IIb was confirmed by the presence of strong absorption band at 2972 cm^{-1}in IR spectrum due to aldehyde C-H stretch. The signal at 9.3 ppm in proton NMR due to aldehyde proton further supports the success of the reaction.

Table - 1:

Physical Data of the Compounds

Sl. No	Compound	Ar*	R_1	Melting point (^{0}C)	Rf Value	% Yield
1	III a	Phenyl	Phenyl	205	0.54	25
2	III b	Phenyl	*m*-tolyl	210	0.64	35
3	III c	Phenyl	*p*-tolyl	190	0.68	40
4	III d	Phenyl	2- pyridyl	190	0.56	25
5	III e	Phenyl	2- thiazolyl	185	0.52	20
6	III f	4-methoxy phenyl	Phenyl	198	0.75	29
7	III g	4-methoxy phenyl	*m*-tolyl	150	0.83	40
8	III h	4-methoxy phenyl	*p*-tolyl	225	0.54	38
9	III i	4-methoxy phenyl	2- pyridyl	196	0.56	25
10	III j	4-methoxy phenyl	2- thiazolyl	182	0.53	20
11	IV a	Phenyl	Dimethylamino	164	0.43	45
12	IV b	Phenyl	Diethylamino	160	0.50	56
13	IV c	Phenyl	Morpholino	162	0.45	56
14	IV d	Phenyl	Piperazino	158	0.50	60
15	IV e	4-methoxy phenyl	Dimethylamino	160	0.64	50
16	IV f	4-methoxy phenyl	Diethylamino	166	0.78	68
17	IV g	4-methoxy phenyl	Morpholino	168	0.92	60
18	IV h	4-methoxy phenyl	Piperazino	160	0.87	65

*For compounds I a & II a - Ar= Phenyl and for I b & II b – Ar = 4-methoxy phenyl

Formation of Schiff base III f from II b was confirmed by ^{1}H NMR spectrum. The absence of singlet due to aldehyde proton at 9.3 ppm, appearance of singlet at 8.37 ppm corresponding to C*H*=N-Ph group of the compound and appearance of additional peaks due to aromatic protons are the evidences suggesting the formation of Schiff base. In the ^{1}H NMR spectrum of Mannich base- IV h, appearance of singlet at 3.69 ´ ppm corresponds to methylene bridge (–N-CH_2-N-)between DHPM and secondary amine moiety, triplet at 2.45-2.6 ppm corresponds to CH_2 of piperazine, singlet at 1.74 ppm due to NH of piperazine indicated the success of the reaction.

The *in vivo* analgesic and anti-inflammatory activities of synthesized compounds were evaluated by Eddy's Hot plate and Carrageenan-induces rat paw oedema method respectively. The compounds were tested at 50mg/kg dose and the results were compared with that of standard diclofenac sodium as a reference drug at the same dose. The results of analgesic activity revealed that compounds III d, III h, IV c and IV h showed significant analgesic activity (76-80%). The results of anti-inflammatory activity indicated that compounds III d and IV c showed maximum anti-inflammatory activity. (52.3% and 87.0% respectively).

Acknowledgement

The authors are grateful to J.S.S Mahavidyapeetha and Dr. H.G.Shivakumar, Principal, J.S.S College of Pharmacy, Mysore for providing research facilities. Authors are thankful to NMR research centre Indian Institute of Science, Bangalore for providing ^{1}H-NMR spectra.

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Studies on Extracellular L-glutaminase production by Halophilic Aspergillus sp.

PRASANTH KUMAR. K[1], PRABHAKAR.T[1*], SATHISH.T[2], GIRIJA SANKAR.G[1], FELEKE MOGES[1], SWARAJYA LAKSHMI.G[1], RAMANA. H[3]

[1]Pharmaceutical Biotechnology Division, A.U.College of Pharmaceutical Sciences, Andhra University, Visakhapatnam - 530003.

[2]Bioengineering and Environmental Centre, Indian Institute of Chemical Technology, Hyderabad-500 007.

[3]Pharmaceutical Chemistry Division, A.U.College of Pharmaceutical Sciences, Andhra University, Visakhapatnam - 530003.

ABSTRACT

L-Glutaminase an enzyme which is used in the treatment of leukemia and also extensively used in the food industry. In the present investigation, glutaminase production pattern was studied under submerged fermentation using isolated halophilic *Aspergillus sp*. The analysis of enzyme production pattern revealed that the maximum production was noticed at pH 6.0, 140 rpm, incubation temperature 28^0C, incubation period 72 hours, 2% inoculum concentration and 2% glutamine concentration. Variation in carbon source supplementation improved the productivity from 22.97 to 27.64 U/ml with 1% (w/v) lactose. Addition of $NaNO_3$ to the fermentation medium improved the glutaminase production. Under the above optimized environment, glutaminase production was improved by 136% with the present microbial isolate.

Key words: glutaminase, leukemia, glutamic acid, submerged, halophilic

Introduction

L-Glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) catalyzes the hydrolysis of L-glutamine to L-glutamic acid and ammonia. This is an essential enzyme for synthesis of various nitrogenous metabolic intermediates. The glutaminase also acts as a catabolic repressor in microorganisms [1]. It is also present in the mammalian tissues and break downs the glutamine into glutamic acid and ammonia [2].

Although L-glutaminase has been reported from many bacterial genera, members of the *Enterobacteriaceae* family have been best characterized. Among bacteria *Escherichia.coli* glutaminases have been studied in much detail. Some examples of bacterial strains producing L-glutaminase include *E.coli, Pseudomonas* species like *P.aeroginosa, P.aureofaciens, P.aurantiaca and P.fluorescens; Acinetobacter* sps*; Bacillus* sps; *Proteus morganii; Proteus vulgaris; Xantomonas juglandis; Erwinia carotovora, E.aroideae, Serratia marcescens, Enterobacter cloacae, Klebsiella aerogens and Aerobacter aerogenes.* Among yeast species *Hansenula, Rhodotorula, Candida scotii, Cryptococcus albidus, Cryptococcus laurentii, Candida utilis, C.nodaensis, Torulopsis candida, Zygosachharomyces rouxii*. Among fungi *Aspergillus oryzae, A.sojae, Tilachlidium humicola, Verticillium althoasei* etc, have been reported to produce glutaminase.

E.coli is the only organism used for industrial applications. Thus seemingly enormous scope exists for the search for potential strains that could produce L-glutaminase in high yields and with novel properties under economically viable bioprocesses. Wade et al, Nelson et al and Imada et al compiled more comprehensive lists of glutaminase producing organisms [3-5].

Literature shows that *Beauveria* sps., could produce extracellular L-Glutaminase even in the absence of an enzyme inducer like L-glutamine or any additional amino acid, when sea water was used in the medium [11]. Considering the potential in vitro applications of glutaminase, the extracellular nature of these enzymes is of much significance from the view point of direct attack upon raw substrate materials and due to the ease of purification. We report the production of extracellular L-glutaminase by halophilic fungi *Aspergillus sp.* under submerged fermentation. The present study envisages optimizing the process and nutritional factors for L-glutaminase production by isolated microorganism.

**Address for correspondence:*
prof.tprabhakar@gmail.com

Materials and Methods

Micro organism and cultural condition

The *Aspergillus* culture used in this study was isolated from marine water of the Bay of Bengal along the Visakhapatnam coast. The production medium was composed of KH_2PO_4 1 g, $MgSO_4$ 0.5 g, $CaCl_2$ 0.1 g, $NaNO_3$ 0.1 g, Trisodium citrate 0.1 g, NaCl 10.0 g, L-glutamine 10.0 g, Dextrose 5.0 g, Sea water 1lit and pH 6.0 After inoculation with a 2% (v/v) conidial suspension, incubated at 27 °C on a rotary shaker at 120 rpm.Initially the fermentation was carried out for 48 hours, the mycelia was separated by centrifugation and the supernatant was used for assaying enzyme activity.

Estimation of glutaminase activity:

Glutaminase was assayed according to the method of Imada et al.where the liberated ammonia due to the action of enzyme was estimated using Nessler's reagent.

Optimization of process parameters for L-glutaminase production

The medium described above was taken as a basal medium and the different process parameters including pH (4 to 9); temperature (26 to 36 °C); rpm (80-180); inoculum concentration (0.5-3.0 % v/v); Glutamine concentration (0.5-3.5% w/v); additional nitrogen sources (0.5 % w/v) viz. peptone, yeast extract, malt extract, ammonium sulphate, meat extract and $NaNO_3$; additional carbon sources (0.5% w/v) viz. lactose, glucose, galactose, starch, maltose and sucrose were optimized independently. Finally the time course of production was evaluated under the optimized conditions. All experiments were conducted in triplicate and the mean values only considered.

Results and Discussion

Any biotechnological product production whether it involves recombinant organisms or by conventional fermentation processes should be economically viable. Optimization of production parameters contributes significantly to the commercial viability of any bioprocess [6]. Hydrogen ion concentration of medium plays vital role in metabolism of any organism and each organism has its optimum pH where growth of selected microbial strain and its cellular metabolism occurs at optimum levels compared to other conditions [7]. Hence, the influence of medium pH on the production of the glutaminase by isolated microbial strain has been investigated by inoculating the strain in the medium having pH range of 4.0 to 9.0 and analyzing the glutaminase production pattern regularly during the fermentation. The data revealed that this microbial strain effectively produces the enzyme in the pH range of 5.0 to 8.0 with maximum production at pH 6.0 indicating its acidic nature (Fig 1). Higher or lower medium pH resulted in lowering of production. The glutaminases are generally

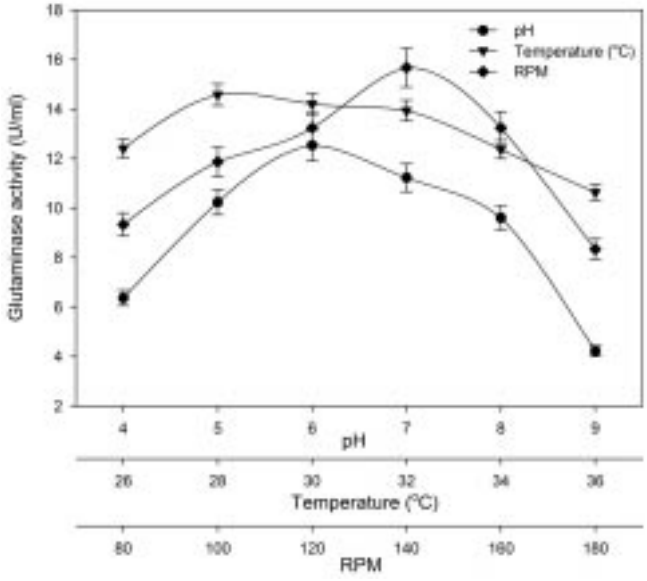


Fig 1: Influence of medium pH, Temperature and RPM on glutaminase production by *Aspergillus sp.*

active at an alkaline pH. While optimal activities of Glutaminase A and B from *Pseudomonas aeruginosa* were at alkaline pH of 7.5-9.0 and 8.5 respectively [8,10]. Glutaminase from *Pseudomonas* sp, was reported to be active over a broad range of pH 5-9 with an optimum near pH 7.

The influence of temperature was studied by carrying out the fermentations between 26 – 36 ^{O}C with the interval of 2^{O}C (Fig 1). Maximum glutaminase production was noticed at 28^{O}C and deviation from this temperature resulted in reduction of enzyme production.

The amount of aeration has a profound effect on the rate of growth which in turn influences enzyme production. Hence, the influence of rotations per minute on glutaminase production was investigated using this microbial strain. Enzyme production increased with increase in rpm during fermentation until 140 rpm and further increase results in decrease of glutaminase production (Fig 1.)

Since microbial productivity is directly proportional to biomass, the role of inoculum level on glutaminase production with this strain was studied. High yield of enzyme was obtained when inoculum concentration was 2% (v/v) and 48 hrs of age was used (Fig 2). It was interesting to note that increase of inoculum level up to 2% resulted in increased glutaminase production and further increase resulted in decreased production. Such inoculum concentration dependent product formation variations were reported in other microbial strains [9].

The concentration of inducers plays a pivotal role in the production of extracellular enzymes. The glutamine acts as an inducer for the production of the glutaminase. In

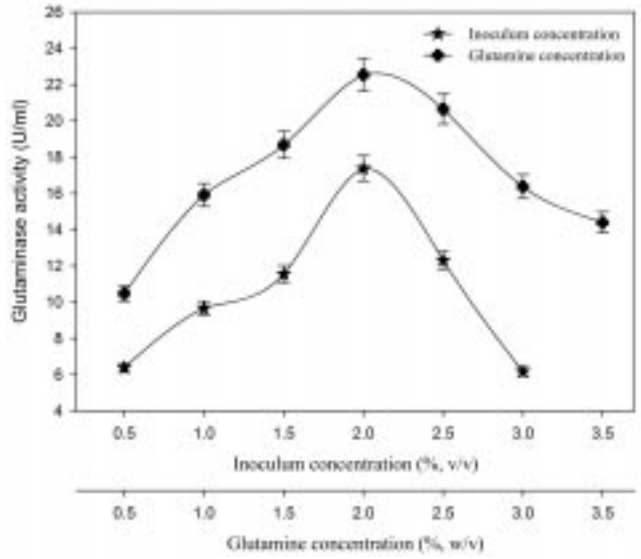
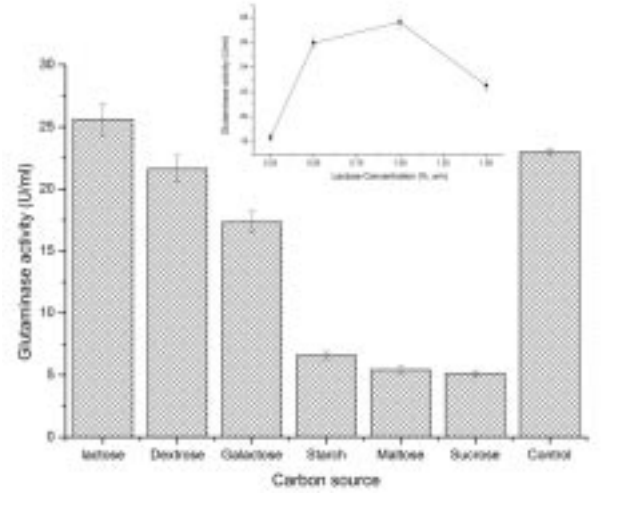


Fig 2: Influence of inoculum and glutamine concentration on glutaminase production by *Aspergillus sp.*

order to find out the appropriate glutamine concentration for optimum glutaminase production the glutamine concentration was varied from 0.5 to 3.5 % in the medium. It was observed that 2% glutamine concentration (Fig 2) gives an increase of 30% in the amount of glutaminase produced. Above or below 2% glutamine concentration decreases the glutaminase production.

Generally the raw materials used as substrates in fermentation processes should be cheap as the profitability greatly depends on the product yield per unit substrate consumed. Thus, although the stoichiometric analysis is the first logical step in process development, it is often difficult to achieve due to the ill-defined nature of the medium. In order to develop an effective medium, the role of different additional carbon (Fig 3) and nitrogen (Fig 4) sources were evaluated for their influence on glutaminase production by this microbial strain. Among the various carbon sources that were screened, lactose was found to be the best and sucrose was the least supporting carbon source for enzyme production (Fig 3 insert). These results are similar to earlier studies on carbon source utilization which showed that the carbon source has a significant effect on growth and product formation. The glutaminase production was studied at different concentrations of lactose and the results indicated that supplementation of 1% lactose into the medium resulted in maximum enzyme production (27.64 U/ml) and an increase or decrease in lactose concentration resulted in reduced production of glutaminase (Fig 3 insert). Among different nitrogen sources studied, $NaNO_3$ enhanced glutaminase production (Fig 4).



(Fig3Insert): Effect of different carbon sources on production of glutaminase by *Aspergillus sp.*, Effect of lactose concentration on glutaminase activity, (Insert).

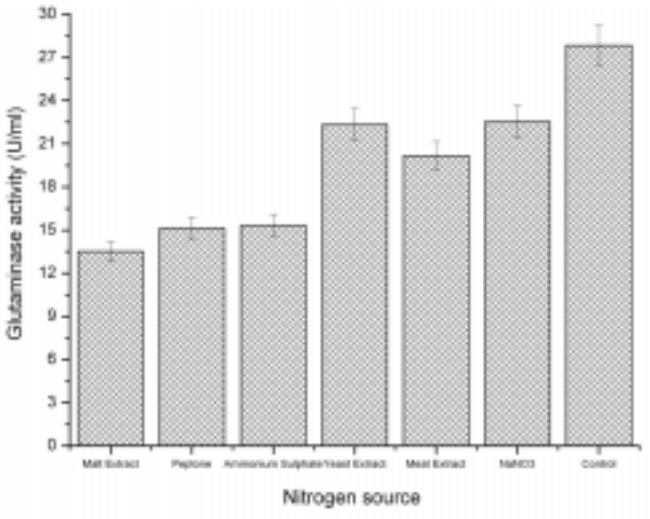


Fig 4: Effect of different nitrogen sources on glutaminase production by *Aspergillus sp.*

The fermentation profile indicated that maximum L-glutaminase production (29.64U/ml) occurred at 72 h of incubation (Fig 5).

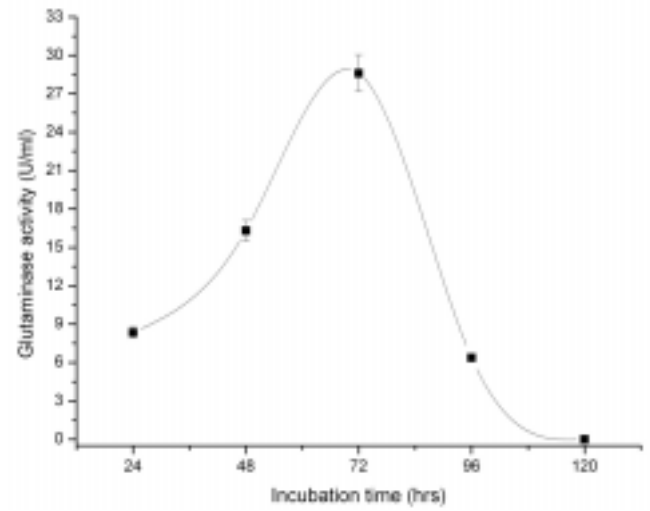


Fig 5: Effect incubation time on production of glutaminase by *Aspergillus sp.*

Further, the maximal L-glutaminase yield (29.64 U/ml) recorded with this fungus is at an appreciable level when compared to earlier reports for fungi (Yano et al. 1988) and bacteria (Renu & Chandrasekaran 1992; Prabhu & Chandrasekaran 1997) [12,13,14]. The present study indicates scope for the use of Aspergillus sp. as an ideal organism for the industrial production of extracellular L-glutaminase.

Conclusion

The potential of isolated *Aspergillus sp.*, for glutaminase production was investigated under different growth and medium conditions. Maximum production was noticed at pH 6.0, at 28^{O}C, 140 rpm, with 2% inoculum, 2% glutamine concentration, 1% lactose as carbon source and 0.5 % $NaNO_3$ at incubation period of 72 hours. Under optimized environment glutaminase production was improved from12.54 to 29.61 U/ml (136%) with this microbial isolate.

Acknowledgements

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Synthesis, spectral studies and antimicrobial activity of metal complexes of 2-methyl-benzoxazole-5-carboxylic acidhydrazones of 2-hydroxy-1-naphthaldehyde and 2-hydroxy-3-methoxybenzaldehyde

RADHAKRISHNA MURTHY CH, RAVINDER M AND SRIHARI S*
Department of Chemistry, Kakatiya University, Warangal – 506 009 (INDIA)

ABSTRACT

The complexes of Cr(III), Mn(II), Fe(III), Co(II), Ni(II), Cu(II), Zn(II), Cd(II) and Hg(II) with hydrazones derived from the condensation reaction of 2-methyl-benzoxazole-5-carboxylic acidhydrazide with 2-hydroxy-1-naphthaldehyde and 2-hydroxy-3-methoxybenzaldehyde have been synthesized and structurally characterized on the basis of analytical, conductance, thermal, magnetic and infrared, electronic and esr spectral data. The ligands behave as mononegative, tridentate ones coordinating through phenolic oxygen, amide group oxygen and azomethine nitrogen. Further, some selected compounds have been screened for their antimicrobial activity and the results are presented.

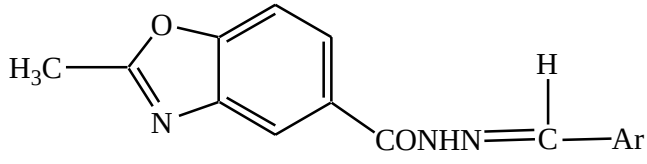
Keywords: benzoxazole acidhydrazone complexes, synthesis, characterization, antimicrobial activity.

Introduction

The chemistry of benzoxazole and its derivatives reported in the literature is interesting due to their biological importance possessing antimicrobial, antiviral, antihistamic, antiinflammatory and antitumor properties [1-5]. In view of the biological importance of this class of compounds, the authors have considered it worthwhile to make use of them as ligands for the preparation of metal complexes and their structural characterization for the characterization studies would throw light on the bonding nature of the ligands and its possible relationship with properties for which the ligands are known. Therefore, we present, herein, the synthesis and structural characterization of Cr(III), Mn(II), Fe(III), Co(II), Ni(II), Cu(II), Zn(II), Cd(II) and Hg(II) complexes of 2-methyl-benzoxazole-5-carboxylic acidhydrazones of 2-hydroxy-1-naphthaldehyde and 2-hydroxy-3-methoxybenzaldehyde abbreviated respectively as MBCHNH and MBCHMH (Fig.1), followed by screening of selected compounds for their antimicrobial activity.

Experimental

All the chemicals used were of AR or BDH grade. The ligands MBCHNH and MBCHMH were synthesized by refluxing an equimolar mixture of 2-methyl-benzoxazole-5-carboxylic acidhydrazide and the respective aldehyde in alcohol (20ml) in presence of a few drops of acetic acid, for about one hour. The product thus obtained was filtered, washed with water, dried and purified by recrystallization from methanol.



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

Fig. 1

The Cr(III), Fe(III) and Hg(II) complexes with the ligands were prepared using respective metal chlorides and Mn(II), Co(II), Ni(II), Cu(II), Zn(II) and Cd(II) complexes using respective metal acetates. In the preparation of all the metal complexes, the metal and the ligand were combined in 1:2 mole ratio (the metal being in slight excess of what the ratio required) using required quantities of methanol or aqueous methanol for the metal salts and methanol for the ligands so as to effect their solubility. The contents were refluxed on a water bath for 2-3 hours and the solid that separated was filtered, washed with water, hot methanol and ether and dried in air.

Address for correspondence

The elemental analyses of the complexes were carried out by C.D.R.I., Lucknow. The metal content in the complexes after decomposition, was determined by standard procedures [6].Conductance measurements were made in DMF at 10^{-3} M concentration on a Digisun digital conductivity meter, DI 909 model. Gouy balance calibrated with $Hg[Co(NCS)_4]$ was used to measure the magnetic susceptibility of metal complexes at room temperature. The IR spectra of the ligands and the metal complexes in KBr were recorded in the range 4000 – 400 cm^{-1} using JASCO FT/IR 5300 spectrophotometer. The electronic spectra of the metal complexes were recorded on Shimadzu UV-Vis spectrophotometer UV-160 A and JASCO 7800 UV-Vis spectrophotometer. The Varian E-4 X-band spectrophotometer operating in the frequency range 8.8 – 9.6 GHz available with R.S.I.C., I.I.T. Chennai was employed in recording ESR spectra of the Cu(II) complexes in DMF at LNT. The compounds were assayed for their antibacterial activity against the bacteria: *Bacillus megaterium* (gram+ve) and *Klebsiella pneumoniae* (gram –ve) by paper disc method [7] and for antifungal activity against fungi: *Fusarium oxysporum* and *Aspergillus niger* by glass humid chamber technique [8].

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 soluble in methanol and acetone and fairly soluble in dimethylformamide.

The Cr(III) and Fe(III) complexes record conductance values in the range 71-75 $ohm^{-1}\ cm^2\ mol^{-1}$ in DMF indicating that they are 1:1 electrolytes. The Mn(II), Co(II), Ni(II), Cu(II), Zn(II), Cd(II) and Hg(II) complexes are, on the contrary, shown to be non-electrolytic with only residual conductance values.

Thermal studies of the selected metal complexes namely Fe(III), Co(II), Ni(II), Cu(II) and Zn(II) complexes of MBCHNH 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, Fe(III) complex above 500^0C, Co(III) complex above 460^0C, Ni(II) complex above 490^0C, Cu(II) complex above 510^0C and Zn(II) complex above 560^0C. 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.

The magnetic studies made on the complexes indicate that Cr(III), Mn(II), Fe(III), Co(II), Ni(II) and Cu(II) complexes are paramagnetic corresponding to three, five, five, three, two and one electrons respectively and that the Zn(II), Cd(II) and Hg(II) complexes are diamagnetic.

IR Spectra

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 1230 cm^{-1} in the ligands assignable to νC-O is seen to have undergone a positive shift by 30-50 cm^{-1} in the complexes suggesting coordination through phenolic oxygen [9]. 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 [10]. The ligands record a somewhat broad, medium intensity band around 3430 cm^{-1} attributable to free νN-H [11]. This band remains either unshifted or higher shifted in the complexes indicating non-participation of nitrogen of this group in coordination. The ligands display a strong absorption band around 1590 cm^{-1} due to νC=O of amide [12]. This band undergoes a lower shift by about 100 cm^{-1} indicating that the oxygen of this group is involved in bonding with the metal ion. A medium intensity band that shows up in the ligands around 1620 cm^{--1} due to νC=N has been found lower shifted by about 20 cm^{-1} in the complexes. The downward shift of this frequency in the complexes suggests involvement of azomethine nitrogen in coordination [13].

The coordination through phenolic oxygen, amide group oxygen and azomethine nitrogen is further substantiated by the appearance, in all the complexes, of non-ligand bands in the far infrared region around 500 and 400 cm^{-1} assignable repectively to νM-O and M-N vibrations [14, 15].

Electronic spectra:

The electronic spectral frequencies observed for the complexes along with liagand field parameters for the Co(II) and Ni(II) compexes are given in Table 2. These frequencies may be assigned, in the increasing order, to the transitions as detailed below [16].

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

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

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

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

Table 1:
Analytical and physical data of metal complexes

Metal complex	Colour	Metal % Found (Cal)	Molar Cond. Ω^{-1} cm^2 mol^{-1}	μ_{eff} B.M.
$[Cr(MBCHNH\ -H)_2]Cl$	Green	6.98 (7.02)	72	3.81
$[Mn(MBCHNH\ -H)_2]$	Black	7.31 (7.39)	12	5.69
$[Fe(MBCHNH-H)_2]Cl$	Brown	6.98 (7.02)	75	5.72
$[Co(MBCHNH-H)_2]$	Green	7.72 (7.88)	14	4.68
$[Ni(MBCHNH-H)_2]$	Reddish brown	7.81 (7.85)	10	2.98
$[Cu(MBCHNH-H)_2]$	Green	8.40 (8.45)	12	1.84
[Zn(MBCHNH -H)OAc]	Light yellow	13.89 (13.95)	13	--
[Cd(MBCHNH-H)OAc]	Yellow	21.71 (21.79)	10	--
[Hg (MBCHNH –H)Cl]	Yellow	34.51 (34.56)	14	--
$[Cr(MBCHMH\ -H)_2]Cl$	Brown	7.01 (7.06)	68	3.80
$[Mn(MBCHMH\ -H)_2]$	Black	7.79 (7.81)	13	5.72
$[Fe(MBCHMH-H)_2]Cl$	Reddish brown	7.49 (7.55)	72	5.70
$[Co(MBCHMH-H)_2]$	Blue green	8.28 (8.33)	16	4.80
$[Ni(MBCHMH-H)_2]$	Yellowish green	8.27 (8.30)	14	3.25
$[Cu(MBCHMH-H)_2]$	Yellowish brown	8.88 (8.92)	12	1.81
[Zn(MBCHMH -H)OAc]	Light yellow	14.49 (14.57)	12	--
[Cd(MBCHMH-H)OAc]	Light yellow	22.62 (22.67)	10	--
[Hg (MBCHMH –H)Cl]	Yellow	35.78 (35.80)	10	--
All the complexes gave satisfactory C,H,N analyses.				

Table 2:
Electronic spectral data of the metal complexes

Metal complex	Frequency (cm^{-1})			10DQ (cm^{-1})	B (cm^{-1})	β
Cr- MBCHNH	16500	23000	32000	---	---	---
Mn- MBCHNH	13120	19180	24610	---	---	---
Fe- MBCHNH	12500	15600	20000	---	---	---
Co- MBCHNH	9300(ν_1)	16220(ν_2)	22980(ν_3)	6918	753	0.775
Ni- MBCHNH	9530(ν_1)	14200(ν_2)	24300(ν_3)	9530	661	0.642
Cu- MBCHNH	15250	20350	---	---	---	---
Cr- MBCHMH	16480	23750	31950	---	---	---
Mn- MBCHMH	13110	19190	25000	---	---	---
Fe- MBCHMH	12410	15550	20450	---	---	---
Co- MBCHMH	9320(ν_1)	16630(ν_2)	22800(ν_3)	7312	765	0.788
Ni- MBCHMH	9270(ν_1)	14010(ν_2)	23950(ν_3)	9270	677	0.657
Cu- MBCHMH	15230	20370	---	---	---	---

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

Cu(II) complex: $^2B_{1g} \rightarrow {}^2B_{2g}$ and $^2B_{1g} \rightarrow {}^2E_g$

Based on these transitions and the other data observed, octahedral geometry for the Cr(III), Mn(II), Fe(III), Co(II) and Ni(II) complexes and tetragonal geometry for Cu(II) complexes have been proposed [17, 18]. Further, the ligand field parameters 10DQ, B and β obtained for the Co(II) and Ni(II) complexes indicate that the metal-ligand bond in them is covalent in nature [19].

ESR Spectra:

The spectra of Cu-MBCHNH and Cu-MBCHMH complexes are of anisotropic nature in that each of them has two peak envelopes, one of small intensity towards low field and the 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). The large intensity peak towards high field has not been resolved. The $g_{\parallel}$ and $g_{\perp}$ values of the complexes indicate that $g_{\parallel} > g_{\perp} > 2$ and so the unpaired electron lies predominantly in the $d_{x^2-y^2}$ orbital with $^2B_{1g}$ as the ground state [20]. The α^2, β^2 and γ^2 values are 0.55-0.59, 0.86-0.93 and 0.65-0.80 suggesting appreciable / moderate / weak in-plane -bonding, in-plane π-bonding and out-of-plane π-bonding respectively [21].

Antimicrobial Activity:

The ligands MBCHNH and MBCHMH and their Fe(III), Cu(II), Zn(II), and Cd(II) complexes have been screened for antimicrobial activity against the bacteria: *Bacillus megaterium* (gram+ve) and *Klebsiella pneumoniae* (gram –ve) and fungi: *Fusarium oxysporum* and *Aspergillus niger*.

The results of the antimicrobial screening of the ligands and their meal complexes are incorporated in Table 3. It may be seen from the table that the activity profiles of the ligands and their metal complexes screened against the microorganisms are varying in that some of the compounds are active while others are not. Further, it may be noted that more of the compounds are active against the fungi than against the bacteria studied and that their antifungal activity is at a higher level compared to their antibacterial activity.

Antibacterial activity: The results on the antibacterial activity of the compounds screened indicate that most of the compounds are ineffective in inhibiting the growth of gram +ve as well as gram –ve bacteria. While Cd-MBCHNH complex is marginally active against gram +ve, the Cd-MBCHMH complex shows marginal activity against both the bacteria.

Antifungal activity: The ligands show marginal activity against the fungi while Cu and Cd complexes of both the ligands are significantly active against fungi; the activity associated with other complexes is marginal.

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.

Table 3:
Antimicrobial activity of the compounds

Solvent: Acetone
Concentration: 0.1 mg/ml

S. No.	Compound	Diameter of growth inhibition zone (in mm)		Percentage of spore germination inhibition (in mm)	
		Bacteria		Fungi	
		Bacillus megaterium (gram+ve)	*Klebsiella pneumoniae* (gram –ve)	*Fusarium oxysporum*	Aspergillus niger
1	MBCHNH	--	--	10.00	6.25
2	MBCHMH	--	--	12.50	8.75
3	Fe- MBCHNH	--	--	13.75	35.00
4	Cu- MBCHNH	--	--	53.75	51.25
5	Zn- MBCHNH	--	--	15	8.75
6	Cd- MBCHNH	2.5	--	60	67.5
7	Fe- MBCHMH	--	--	17.50	6.25
8	Cu- MBCHMH	--	--	43.75	38.75
9	Zn- MBCHMH	--	--	18.75	11.25
10	Cd- MBCHMH	2.5	3.0	62.50	75.00

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Rapid synthesis and evaluation of some azetidinone and thiazolidinone derivatives of coumarins

SIRISHA. K[2], SHIVA RAMA KRISHNA. B[1], ACHAIAH. G[2] & REDDY, V.M[1*]
[1]Medicinal Chemistry Division, Vaagdevi College of Pharmacy, Ram Nagar, Hanamkonda, Warangal – 506 001 (A.P.)
[2]University College of Pharmaceutical Sciences, Kakatiya University, Warangal – 506 009 (A.P.)

ABSTRACT

A simple, fast and efficient method was developed for the synthesis of 3-chloro-4-(substituted)-1-(3'-carboxamido coumarinyl)-2-azetidinones (**7a-e**) and 2-(substituted)-3-(3'-carboxamido coumarinyl)-1,3-thiazolidinones (**8a-e**) from the corresponding N-(substituted arylidene/heterylidene)imino-3-carboxamido coumarins (**6a-e**).Condensation of compounds (**6a-e**) with chloroacetyl chloride and triethylamine yielded the respective azetidinones (**7a-e**) whereas their condensation with mercaptoacetic acid in presence of anhydrous zinc chloride afforded the respective thiazolidinones (**8a-e**). The required Schiff bases (**6a-e**) were prepared by the condensation of coumarin-3-acid hydrazide (**4**) with five different aromatic / heteroaromatic aldehydes. Compounds **7** and **8** were also prepared by rapid and improvised (MWI) methods using DMF as the solvent. Remarkable reduction in time with significantly increased yields were recorded when compared with conventional methods. In general, the compounds were characterized based on their analytical and spectral (IR, ^{1}H NMR) data and in the case of the compounds **3** and **4** mass spectra were also used. The title compounds (**7 & 8**) were evaluated *in vitro* for their antibacterial activity by a standard method and found some of the compounds could show activity at higher concentrations when compared with the standard.

Key words : Azetidinones, thiazolidinones, Schiff base, coumarin, antibacterial activity.

Introduction

The increasing application of microwave irradiation as a source of thermal energy in organic reactions [1] is due to the short reaction time and the operational simplicity. The use of domestic microwave oven in this regard is now a well established procedure in MORE [2] (Microwave induced Organic Reaction Enhancement) chemistry. It can be termed [3] as 'e-chemistry' because it is easy, effective, economical and eco-friendly and is believed to be a step towards green chemistry.

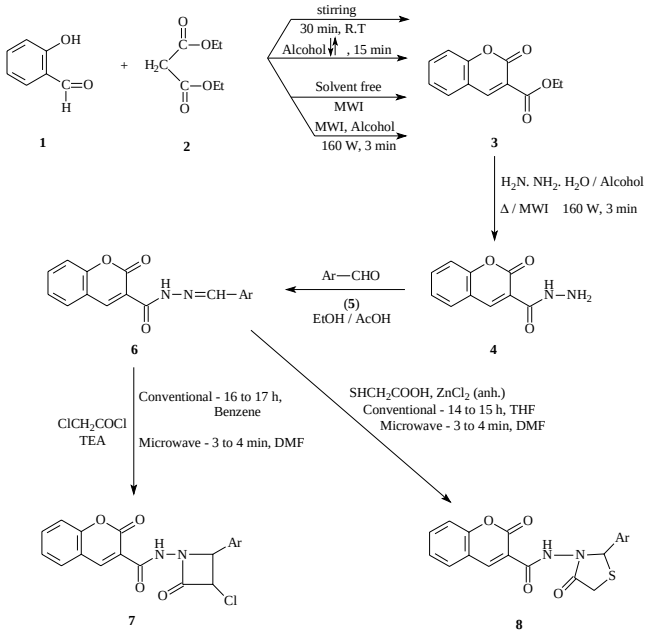
Azetidinones and thiazolidinones are well known for their biological activities such as antimicrobial [4,5], anticancer [6,7] and antidiabetic [8,9]. Furthermore, coumarins are biologically active, synthetically useful and important heterocycles having wide role in medicinal chemistry [10-14]. Thus, with an effort to capitalise the pharmacological potential of the above heterocyclic nuclei and to synthesize biologically potential compounds, it was designed to bring together such important molecular moieties into a single molecular frame work. The details of the pertinent work are presented in this communication.

**Address for correspondence:*

The Knoevenagel condensation of salicylaldehyde **(1)** with diethyl malonate **(2)** in presence of a trace amount of piperidine was carried out under both the reported experimental conditions [15-17] and improvised conditions to get coumarin-3-carboxylic acid ethyl ester **(3)**. The coumarin ester **(3)** upon hydrazinolysis with hydrazine hydrate in alcohol under microwave irradiation led to the formation of coumarin-3-carboxylic acid hydrazide **(4)**. Though the preparation of ethyl coumarin-3-carboxylate and its acid hydrazide were previously reported by some authors [18-21], the reported data appeared to be quite inconsistent with respect to reaction conditions, yields, melting points, spectral data etc. which was leading to utter confusion. Therefore, it was thought to re-investigate such reactions under reported conditions to verify the truth and also to improvise them if possible.

A condensation reaction of compound **4** with various aldehydes **(5)** in aceticacid furnished the corresponding 3-N-(substituted arylidene/heterylidene) imino-3-carboxamido coumarins (**6a-e**).A cycloaddition reaction of the Schiff bases (**6a-e**) with chloroacetyl chloride in presence of DMF and triethylamine afforded 3-chloro-4-(substituted)-1-(3'-carboxamidocoumarinyl)-2-azeti-dinones (**7a-e**) and a similar reaction with mercaptoacetic acid in presence of DMF and

anhydrous zinc chloride furnished 2-(substituted)-3-(3'-carboxamido coumarinyl)-1, 3-thiazolidinones (**8a-e**) **(Scheme-1).**



Scheme-1

The microwave procedures for the synthesis of the title compounds viz., azetidinones (**7**) and thiazolidinones (**8**) owe their importance to the fact that the reaction could be completed within 3-4 min as compared to 16-17 h heating in conventional method.

Materials and methods

Melting points of the compounds were determined in open capillaries using Toshniwal and Cintex melting point apparatus and are uncorrected. IR spectra of the compounds were obtained as KBr pellets on Perkin-Elmer spectum BX-I series, FT-IR spectrophotomer, ^{1}H NMR spectra on Gemini 300 MHz spectrophotometer, and mass spectra of the compounds were recorded on Micromass Quatro II Mass spectrophotometer operating in the ESI mode (70 eV). Progress of every reaction was monitered by TLC. Elemental analyses were carried out using the EURO-EA analyzer. Microwave assisted reactions were carried out in a domestic microwave oven (LG, Model No. MS-2342 AE).

Preparation of ethyl coumarin-3-carboxylate (3)

Method – I : Stirring at ambient temperature

A mixture of salicylaldehyde (1.04 ml, 1.22 g, 0.01 moles) in diethyl malonate (1.52 ml, 1.6 g, 0.01 moles) was treated with piperidine (1 ml, 0.85 g, 0.01 moles) and stirred for 30 min, at room temperature. Then it was acidified with 10% HCl while cooling. The precipitated compound was filtered, washed with small portions of cold water and dried. The product was purified by recrystallization from ethyl acetate (Yield : 42%; m.p .90-92^{o}C) [17].

Method-II : Heating under reflux

Salicylaldehyde (1.04 ml, 1.22 g, 0.01 moles) and diethyl malonate (1.52 ml, 1.6 g, 0.01 moles) were dissolved in alcohol to give a clear solution. Few drops of piperidine were added and heated under reflux for 15-20 min. The reaction mixture was cooled, the crystalline product separated out was filtered and washed with small portions of ice cold aqueous alcohol. Recrystallization was effected using ethyl acetate (Yield : 55% m.p. 90-92^{o}C) [15].

Method-III : Solvent free microwave irradiation :

A mixture of salicylaldehyde (1.04 ml, 1.22 g, 0.01 moles), diethylmalonate (1.67 ml, 1.75 g, 0.011 moles) and piperidine (0.25 ml, 0.003 moles) was irradiated in a microwave reactor at 160 W for 3 min. Then, the reaction mixture was cooled to room temperature and the crude product was recyrstallized from ethyl acetate to afford the coumarin ester (Yield : 74% ; m.p, 92-93^{o}C) [16].

Method IV : Microwave irradiation in solvent (alcohol) :

Salicylaldehyde (1.04 ml, 1.22 g, 0.01 moles), diethyl malonate (1.52 ml, 1.6 g, 0.01 moles) and piperidine (0.25 ml, 0.003 moles) were dissolved in 10 ml alcohol and irradiated in the microwave oven at 160 W power for 3 min. Then, the reaction mixture was cooled in a refrigerator, and the crude product obtained was filtered, washed with a small portion of ice cold alcohol. Recrystallization was effected using a mixture of n-hexane : benzene (1:1) or pet ether : benzene (1:1) to afford the pure product (Yield : 82%, m.p. 92-94^{o}C).

3 : IR (KBr) : 3064.9 (C-H str, arom), 2966-2979 (C-H str, aliph), 1770 (C=O, α, β-unsatd, γ-lactone), 1735 (C=O, ester), 1617 (C=C of α, β-unsatd. Ketone), 1607 (C=C, aromatic), 1564 (C=C, aliph), 1164 (C-O str of benzopyran).

^{1}H NMR ($CDCl_3$) : 1.42 (t, 3H, J = 7.1 Hz, -CH_2-CH_3), 4.4 (q, 2H, J = 7.1 Hz, -$OCH_2$0CH_3), 7.32 (dd, 2H, J=7.6, Ar-H at C_6 & C_8), 7.62 (dd, 2H, J=7.62, Ar-H at C_5 & C_7) and 8.48 (s, 1H, C_4-H). MS : m/z 219 (M+1) (100%), 173 (28%), 146, 118, 102, 77, 89, 63.

Preparation of coumarin-3-acid hydrazide (4)

Method-I : Heating under reflux

Coumarin ester (**3,** 2.18 g, 0.01 moles) was dissolved in alcohol and hydrazine hydrate (99%, 0.75 g, 0.015 moles) was added and heated under reflux for 10-12 h. Excess of solvent was distilled-off. Crystalline solid separated was filtered, washed and recrystallized from alcohol (Yield : 64%; m.p. 146-148^{o}C) [21].

Method II : MWI in alcohol

A mixture of coumarin-3-carboxylic acid ethyl ester (**3**, 2.18g, 0.01 moles) and hydrazine hydrate (99%, 0.75 g,

0.015 moles) in alcohol (10 ml) was irradiated in a microwave oven for 3 min, at 160 W. Then, the reaction mixture was cooled to obtain an yellow crystalline solid, which was purified by recrystallization from alcohol (Yield : 72%, m.p. 148-150°C).

4 : IR (KBr) (γ, cm^{-1}) : 3305.37 (N-H str), 1698 (γ-lactone) and 1654.15 (C=O, str), 1364.03, 1053.66, 668.35. ^{1}H NMR ($CDCl_3$) : 11.39 (s, 1H, NH), 8.71 (s, 1H, H4), 7.42 (d, 1H, H5), 7.39 (t, 1H, H7), 7.34 – 7.39 (m, 2H, H6 and H8), 7.26 (s, 2H, NH_2). MS : m/z 204 (M^+), 189 (6%), 154 (16%), 121 (100%), 69 (39%).

Preparation of 3-N-(Aryl)imino-3-carboxamido coumarin (6)

Method – I : General Procedure :

A mixture of coumarin-3-acid hydrazide (**4**, 1.02 g, 0.005 moles) and appropriate aromatic aldehyde (**5,** 0.005 moles) was taken in a dry beaker (100 ml). To this freshly distilled glacial acetic acid (10 – 15 ml) was added and the reaction mixture was stirred constantly to get a clear solution. It soon started yielding a product. It was then poured into ice cold water and the product resulted was filtered, washed with cold water and dried. The compound was purified by recrystallization from alcohol.

Method-II : General Procedure :

A mixture of coumarin-3-acid hydrazide (**4,** 1.02 g, 0.005 moles) and an appropriate aromatic aldehyde (**5,** 0.005 moles) was taken in a dry beaker (100 ml). To his, dry alcohol (15 ml) and few drops of freshly distilled glacial acetic acid were added and the reaction mixture was warmed on a water bath until a clear solution was obtained. It was then cooled, poured onto ice-cold water and the Schiff base obtained was filtered, washed with cold water, dried and recrystallised from alcohol. (The yield of Schiff base in both the methods was found to be almost similar).

Following the above procedures, compound **6a-e** were prepared and their characterization data are presented in **Table -1.**

Preparation of 3-chloro-4-(aryl)-1-(3'-carboxamidocoumarinyl) -2-azetidinone (7)

Conventional method : General Procedure :

The Schiff base (**6,** 0.0025 moles) in benzene was taken in a round bottomed flask (50 ml). To it chloroacetyl chloride (0.03 mL, 0.0025 moles) and triethyl amine (0.37 ml, 0.0025 moles) in benzene were added slowly while cooling in an ice bath. The mixture was then heated under reflux for 16-17 h while monitoring by TLC. After completion of the reaction, the solvent was distilled of under reduced pressure and the residue was triturated with crushed ice (50 g). The resultant product was filtered, washed with cold water and dried. It was purified by recrystallization from alcohol.

Table 1:
Characterization data of Schiff Bases (6a-e)

Compound	Substituent Ar	m.p. °C	Method I Yield (%)	Method II Yield (%)	Mol. formula (Mol. wt)	Found (Calcd) (%)			IR (KBr, cm^{-1})
						C	H	N	
6a	-C_6H_5	214-216	90	85	$C_{17}H_{12}N_2O_3$ (292.286)	69.86 (69.85	4.14 4.13	9.50 9.58)	--
6b	p-$CH_3OC_6H_4$	196-198	92	87	$C_{18}H_{14}N_2O_4$ (322.312)	67.07 (67.01	4.30 4.37	8.65 8.68)	3214.43 (N-H str), 3077.67 (Arom C-H, Str), 1652.11 (C=O Str), 1558.48 (N-H bend), 1240.01 (Asym C-O-C str) 756.20 (out of plane C-H bend)
6c	p-OH, m-$CH_3OC_6H_3$	216-218	70	65	$C_{18}H_{14}N_2O_5$ (338.312)	63.75 (63.77	4.18 4.16	8.20 8.26)	3450.30 (N-H str), 3178.88 (Ar O-H str), 1684.01 (C-O str), 1599.67 (C=C str), 1516.81 (NH bend), 1270.03 (asym C-O-C str), 1031.98 (sym C-O-C str), 751.88 (out of plane C-H bend)
6d	Furfuryl	222-224	85	82	$C_{15}H_{10}N_2O_4$ (282.250)	63.79 (63.77	3.53 3.56	9.87 9.91)	--
6e	3-Chromone	218-220	75	70	$C_{20}H_{12}N_2O_5$ (360.316)	66.65 (66.66	3.35 3.35	7.77 7.76)	--

Table 2:
Characterization data of Azetidinone compounds (7a-e)

Compound	m.p. °C	Yield (%) (Period/hr) Conventional method	Yield (%) (Period/min) Microwave method	Mol. formula (Mol. wt)	Found (Calcd) (%)		
					C	H	N
7a	168-170	62 (16.0)	69 (3.0)	$C_{19}H_{13}N_2O_4Cl$ (368.764)	61.82 (61.87	3.53 3.55	7.54 7.59)
7b	150-156	60 (18.0)	62 (4.0)	$C_{20}H_{15}N_2O_5Cl$ (398.790)	60 (60.05	3.79 3.78	7.05 7.00)
7c	148-152	72 (17.0)	76 (4.0)	$C_{20}H_{15}N_2O_6Cl$ (414.790)	57.92 (57.90	3.65 3.64	6.76 6.75)
7d	160 (d)	70 (16.0)	75 (4.0)	$C_{17}H_{11}N_2O_5Cl$ (358.728)	56.93 (56.91	3.05 3.08	7.75 7.78)
7e	180-184	65 (18.0)	70 (4.0)	$C_{22}H_{13}N_2O_6Cl$ (436.794)	60.25 (60.24	2.99 2.98	6.35 6.38)

IR : 7b : 3213.85 (N-H Str), 3066.33 (Arom C-H str), 1654.91 (C=O Str), 1604.29 (C=C str), 1511.46 (N-H bend), 1251.73 (asym C-O-C str), 1027.78 (sym C-O-C str)

HNMR 7b: ($CDCl_3$,´ ppm) : 3.72 (s,3H,Ar-OCH_3),6.80(d,1H,-N-CH-Ar),6.92(d,1H,-CO-CH-Cl),7.50-7.92(m,8H,Ar-H) 8.50 (s,1H,Ar-H) and 11.08 (s,1H,-CO-NH)

Microwave method : General Procedure :

The Schiff base (**6,** 0.0025 moles) in DMF (10 ml) was taken in a beaker (100 ml) and added slowly chloroacetyl chloride (0.3 ml, 0.0025 moles) and triethyl amine (0.37 ml, 0.0025 moles) while cooling in an ice-bath. An inverted funnel was placed at the rim of the beaker and the contents were irradiated in a microwave oven at 640 W for 3-4 min. The reaction mixture was cooled, poured onto crushed ice (~100 g) while stirring. The resultant solid product was filtered, washed with cold water and dried. It was purified by recrystallization from alcohol.

Following the general procedure, the compounds **7a-e** were prepared and characterized. The peritent data are shown in **Table-2**.

Preparation of 2-Aryl-3-(3'-carboxamidocoumarinyl)-1,3,-thiazolidinone (8):

Conventional method : General Procedure :

A mixture of Schiff base (**6**, 0.0025 moles) in THF (30 ml), mercaptoacetic acid (0.02 ml, 0.0025 moles) and a pinch of anhydrous zinc chloride was heated under reflux on a water bath for 15 h while monitoring by TLC. The reaction mixture on cooling yielded a product which was washed first with cold $NaHCO_3$ solution and then extensively with cold water and dried. It was purified by recrystallization from dioxan .

Microwave method : General Procedure :

A mixture of Schiff base (**6**, 0.0025 moles). in DMF (15 ml) and mercaptoacetic acid (0.02 ml, 0.0025 moles) and a pinch of anhydrous zinc chloride was irradiated in a microwave oven at 640 W for 3-4 min while monitoring by TLC. The reaction mixture was cooled and poured onto crushed ice (~100 g) while stirring. The solid separated was filtered, washed with portions of cold water and dried. The product was purified by recrystallization from dioxan.

Following the general procedure, the compounds **8a-e** were prepared and then characterized. The characterization data of **8a-e** are presented in **Table-3**.

Antibacterial activity

The compounds were tested for their possible antibacterial activity by the cup plate technique [22], against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Proteus vulgaris* as test organisms. The compounds possess moderate to good activity against all stains in comparison with streptomycin. The test compounds were dissolved in DMF solvent and the zones of inhibition (mm) were measured after 24h incubation at 37ÚC and the obtained data is presented in **Table-4**.

Table 3:
Characterization data of Thiazolidinone compounds (8a-e)

Compound	m.p. °C	Yield (%) (Period/hr) Conventional method	Yield (%) (Period/min) Microwave method	Mol. formula (Mol. wt)	Found (Calcd) (%)		
					C	H	N
8a	216-220	65 (15.0)	70 (3.0)	$C_{19}H_{14}N_2O_4S$ (366.382)	62.10 (62.06	3.85 3.83	7.66 7.62)
8b	196-200	62 (16.0)	72 (3.0)	$C_{20}H_{16}N_2O_5S$ (396.408)	60.55 (60.59	4.06 4.06	7.10 7.06)
8c	210 (d)	70 (15.0)	75 (4.0)	$C_{20}H_{16}N_2O_6S$ (412.408)	58.15 (58.12	3.89 3.90	6.75 6.78)
8d	184-186	68 (14.0)	70 (2.5)	$C_{17}H_{12}N_2O_5S$ (356.326)	57.15 (57.16	3.35 3.38	7.90 7.84)
8e	198-202	60 (17.0)	65 (4.0)	$C_{22}H_{14}N_2O_6S$ (434.412)	60.66 (60.70	3.25 3.24	6.43 6.44)

IR : 8e : 3318.45 (N-H str), 3128.93 (Arom O-H str), 1664.04 (C=O str), 1621.30 (N-H bend), 1272.06 (asym. C-O-C str), 746.72 (out of plane C-H bend)

^{1}HNMR 8e (DMSO-d_6, δ ppm): 3.24 (s, 2H, -CH_2 of thiazolidinone), 5.65 (s, 1H, -CH of thiazolidinone), 6.62-7.20 (m, 5H, Ar-H), 7.21-7.55 (m, 4H, Ar-H), 8.90 (s, 1H, Ar-H), 11.10 (s, 1H, N-H)

Results and Discussion

A pragmatic approach to the synthesis of a new series of 3-Chloro-4-(substituted)-1-(3'-carboxamido coumarinyl)-2-azetidinones **(7a-e)** and 2-(substituted)-3-(3'- carboxamido coumarinyl)-1,3-thiazolidinones **(8a-e)** has been presented in this communication. Though the synthesis of some other coumarinyl thiazolidinones and azetidinones were reported by conventional methods, but so far not on the present type of compounds. Further, it is for the first time the rapid, MWI method has been made use ,in this synthesis.The advantages of this novel synthetic route are: improved yields of the cyclized products, easy to handle the reaction processes, less time consumption and less hazardous(eco-friendly).

The antibacterial data of the compounds**(7a-e)** and **(8a-e)** are summarized in **Table-4** together with the data of the standard drug streptomycin. It could be noted that though the antibacterial activity of the test compounds is not comparable with that of the standard, they could exhibit a reasonably potent activity at relatively higher concentration.

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Table 4:
Antibacterial data of azetidinones (7a-e) and thiazolidinones (8a-

Compound	Conc. (ppm)	Zone of inhibition (in mm)			
		E. coli	*S. aureus*	*B. subtilis*	*P. vulgaris*
7a	500	12	15	14	15
	1000	14	16	16	17
	1500	15	17	17	18
7b	500	18	17	14	15
	1000	20	17	15	16
	1500	24	21	16	18
7c	500	19	18	16	12
	1000	22	20	18	14
	1500	23	22	18	16
7d	500	16	17	13	17
	1000	16	18	15	18
	1500	17	20	16	19
7e	500	14	15	12	15
	1000	16	17	14	16
	1500	18	18	15	17
8a	500	14	15	15	13
	1000	16	16	17	14
	1500	17	17	17	15
8b	500	10	12	13	13
	1000	15	15	14	14
	1500	16	18	15	15
8c	500	14	15	14	16
	1000	15	16	15	18
	1500	15	17	17	18
8d	500	14	15	14	14
	1000	15	15	15	16
	1500	16	16	15	18
8e	500	14	12	13	11
	1000	15	14	15	13
	1500	15	14	17	15
Streptomycin	100	22	20	19	18

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Synthesis and Antimicrobial Activity of Some oxadiazole derivatives containing Quinoline moiety

*SUNIL KUMAR CHAITANYA P, ISHWAR BHAT K, IRFAN ALI. MD
Department of Pharmaceutical Chemistry, NGSM Institute of Pharmaceutical Sciences, Mangalore-574160, Karnataka, India.

ABSTRACT

2-[(2-methyl quinolin-8-yl)oxy]acetohydrazide (2) was condensed with different aromatic aldehydes to give arylidene derivatives (3a-i) which on cyclization in the presence of yellow mercuric oxide and iodine yielded a new series of 1,3,4-oxadiazoles (4a-i).The new series of compounds were charecterized by spectral data. Most of the compounds tested have shown promising antibacterial and antifungal activity.

Keywords: Oxadiazoles, antibacterial, antifungal.

Introduction

In continuation of our work on synthesis of heterocyclic compounds containing quinoline nucleus ,we report in this paper , synthesis and antimicrobial studies of some 2,5-disubstituted 1,3,4- oxadiazoles containing quinoline moiety.

2,5-disubstituted-1,3,4-oxadiazole nucleus has been reported for antibacterial, antifungal [1-7],anticancer [8], insecticidal[9], anti-inflammatory[10,11], CNS stimulant [12] and antihypertensive [13] activities. Similarly quinoline nucleus appears in natural products such as cinchona alkaloids. The quinoline nucleus is present in flouro quinolone group of drugs. In view of the above observations and in continuation of our studies on heterocyclic compounds of biological interest we have synthesized a new series of oxadiazoles carrying quinoline moiety and screened for their antibacterial and antifungal activities.

In the present work 2-methyl,8-hydroxy quinoline when treated with ethylchloroacetate in presence of anhydrous Potassium carbonate in dry acetone yielded ethyl[(2-methylquinolin-8-yl)oxy]acetate **1** .Hydrazinolysis of **1** with hydrazine hydrate yielded 2-[(2-methylquinolin-8-yl)oxy]acetohydrazide **2**.Condensation of **2** with various substituted aromatic aldehydes yielded arylidene derivatives(3a-3i) , which on cyclization in the presence of iodine and yellow mercuric oxide afforded 8-{{5-(substituted phenyl)-1,3,4-oxadiazol-2-yl]-methyl}-2- methyl quinoline(4a-4i).

*Address for correspondence:
sunilchaitanya@yahoo.co.in

The structures of the newly synthesized compounds were assigned on the basis of IR, ^{1}HNMR and Mass spectral analysis. The compounds were screened for their in-vitro antibacterial and antifungal activities.

Results and Discussion:

Synthesis of oxadiazole derivatives by the described method resulted in products with good yield. All the reactions were carried out under prescribed laboratory conditions. The solvents and reagents used in synthetic work were of laboratory grade and were purified by distillation.

Purity of the newly synthesized compounds was determined by melting point by open capillary method .Progress of the reactions was monitored by TLC.

Biological Activity

Antibacterial activity

Evaluation of the antibacterial activity of the synthesized compounds has been carried out against four pathogenic organisms,viz., *Stapphylococcus aureus, Bacillus subtilis, Eshcerichia coli* and *Pseudomonas aureuginosa*. The antibacterial activity of the newly synthesized compounds in the present study was assessed by cup-plate method. The results of the antibacterial studies are shown in table 2. Among the compounds tested 4d , 4e and 4i have shown good activity against all the pathogens. Compound 4c,4f and 4g have not shown any activity against *P.aeuriginosa*. Ciprofloxacin (10μg) was used as the standard drug. All the compounds were tested at 50 μg level.

I. Physical data of the compounds(3a-3i)

Compound	R	Molecular formula	Molecular weight	Melting point °c	R_f	%yield
3a	C_6H_4-4-OH	$C_{19}H_{16}N_3O_3$	334	142-144	0.78	80
3b	C_6H_4-4-N$(CH_3)_2$	$C_{21}H_{22}N_4O_2$	362	110-112	0.71	65
3c	C_6H_4-3-OH	$C_{19}H_{16}N_3O_3$	334	156-158	0.85	62
3d	C_6H_4-3-NO_2	$C_{19}H_{16}N_4O_4$	370	147-148	0.91	60
3e	C_6H_4-4-Cl	$C_{19}H_{16}N_3O_2Cl$	352	132-134	0.51	52
3f	C_6H_3-3,4-$(OCH_3)_2$	$C_{21}H_{21}N_3O_4$	380	160-162	0.78	79
3g	Furfuryl	$C_{17}H_{13}N_3O_3$	308	130-132	0.56	54
3h	C_6H_4-4-OCH_3	$C_{20}H_{18}N_3O_3$	336	152-154	0.68	56
3i	C_6H_4-4-NO_2	$C_{19}H_{16}N_4O_4$	370	158-160	0.80	72

I. Physical data of the compounds (4a-4i)

Compound	R	Molecular formula	Molecular weight	Melting point °c	R_f	%yield
4a	C_6H_4-4-OH2$C_{19}H_{16}N_3O_3$	333	174-176	0.62	60	
4b	C_6H_4-4-N$(CH_3)_2$	$C_{21}H_{22}N_4O_2$	361	142-144	0.67	54
4c	C_6H_4-3-OH	$C_{19}H_{16}N_3O_3$	333	162-164	0.73	56
4d	C_6H_4-3-NO_2	$C_{19}H_{16}N_4O_4$	369	172-174	0.68	58
4e	C_6H_4-4-Cl	$C_{19}H_{16}N_3O_2Cl$	351	146-148	0.74	50
4f	C_6H_3-3,4-$(OCH_3)_2$	$C_{21}H_{21}N_3O_4$	379	154-156	0.89	61
4g	Furfuryl	$C_{17}H_{13}N_3O_3$	307	183-185	0.79	52
4h	C_6H_4-4-OCH_3	$C_{20}H_{18}N_3O_3$	335	124-126	0.60	53
4i	C_6H_4-4-NO_2	$C_{19}H_{16}N_4O_4$	369	188-190	0.56	68

II. Anti microbial data of the compounds

Compound		Diameter of zone of inhibition			
	B.subtilis	*S.aureus*	*E.coli*	*P.aeruginosa*	*C.albicans*
4a	16	19	22	14	14
4b	14	18	21	12	-
4c	15	19	20	-	-
4d	18	22	23	17	15
4e	19	21	24	18	16
4f	15	13	17	-	-
4g	14	14	15	-	10
4h	15	14	18	16	13
4i	17	18	22	18	14
Ciprofloxacin	19	22	24	20	-
Griseofulvin	-	-	-	-	17

Antifungal activity:

The antifungal activity studies of the synthesized compounds have been carried out against the fungus *Candida albicans* by cup-plate method. Among the compounds tested 4a, 4d and 4i showed good activity and compounds 4h and 4g showed moderate activity. Compounds 4b and 4c and 4f did not showed any activity. Griseofulvin was used as standard drug (10 µg). All the compounds were tested at 50 µg level.

Experimental

TLC (methanol:benzene as mobile phase) was used to monitor the progress of the reaction. The melting points of the newly synthesized compounds were determined in open capillaries and were uncorrected. The IR spectra were recorded on Perkin Elmer IR spectrometer. The ^{1}HNMR spectra were recorded on Bruker AC 300F (300MHz) NMR spectrometer using DMSO / $CDCl_3$ as solvent and TMS as internal standard. All chemical shift values are expressed in the δ scale downfield from TMS and proton signals are indicated as s=singlet, d=doublet, t=triplet and m = multiplet. Mass spectra of the compounds were recorded on a Jeol-D300 mass spectrometer by operating at 70 ev

Procedure

Synthesis of ethyl{(2-methylquinolin-8-yl)oxy}acetate(1)

A mixture of 2-methyl,8-hydroxyquinoline (0.05mol), ethylchloroacetate (0.05mol) and anhydrous potassium carbonate in dry acetone was refluxed for 24 hours on water bath at 70°C. The resultant reaction mixture was cooled and filtered. The excess solvent was distilled off and the reaction mixture was dissolved in ice cold water. Further extracted with ether and the ethereal layer was washed with cold water and dried over anhydrous sodium sulphate. The ether portion was concentrated to get the corresponding ester.

IR(Cm^{-1}): 3053.83 (CH),1755.26 (C=O, ester),1249.05(C-O-C) 1HNMR (δPPM): 7.306.87(5H,m,Ar), 4.86(2H,s,OCH_2), 4.20 (2H,m, CH_2, ethyl), 2.67 (3H , s , CH_3) , 1.23 (3H , m, C H_3 , ethyl) .

Synthesis of 2 - [(2 - methylquinolin - 8 - yl) oxy] acetohydrazide (2)

A mixture of **1** (0.05mol) and hydrazine hydrate99% (0.07mol) in ethanol was refluxed for 6 hours. The excess of solvent is distilled off and the separated product was recrystallized from ethanol.

IR(Cm^{-1}): 3369.1(NH), 3067.5(CH), 1662.6 (C=O, amide), 1425.7(C-N) 1HNMR(δ PPM): 10.64(1H,s,NH), 8.07-7.12(5H,m,Ar),4.82(2H,s,OCH_2) 3.73(2H,m,NH_2), 2.80(3H,s,CH_3).

Synthesis of N-(substituted benzylidene)-2-(2-methyl quinolin-8-yl oxy)acetohydrazide (3a-i):

A mixture of **2** (0.01mol) and substituted aromatic aldehyde (0.01mol) was refluxed along with a few drops of glacial acetic acid for 6 hours. The reaction mixture was cooled and then poured on to crushed ice and stirred well. The separated solid was filtered and recrystallized from ethanol.

Synthesis of2-(substituted phenyl)-5-(2-methyl quinolin-8yl) methoxy)-[1,3,4]-oxadiazole (4a-i):

A solution of **3** (0.01mol) in DMF (60ml) was stirred with yellow mercuric oxide (6gm) and iodine (3gm) at room temperature for 48 hr under anhydrous condition. The reaction mixture was filtered and the filtrate was poured into crushed ice. The separated solid was washed with water and recrystallized from suitable solvent.

Spectral data

N-(4-hydroxy benzylidene)-2-(2-methyl quinolin-8-yl oxy)acetohydrazide (3a):

IR(cm^{-1}) ,3387.63 (OH),3193.95(NH),2987.30 (CH), 1687.31(C=O of amide).

^{1}HNMR(DMSO): 11.43(1H,s,NH),8.4(1H,s,-N=CH),8.52-6.85(9H,m,C-3,C-4,C-5,C-6,C-7 protons of quinoline and 4 Ar-H),4.85(2H,s,OCH_2),2.76 (3H,s,CH_3).

N-(4-metoxy benzylidene)-2-(2-methyl quinolin-8-yl oxy)acetohydrazide (3h):

IR(cm^{-1}) ,3280.41 (NH),3073(CH),1671.24(C=O of amide).

^{1}HNMR(DMSO):11.01(1H,s,NH),8.60(1H,s,-N=CH),8.33-6.85(9H,m,C-3,C-4,C-5,C-6,C-7 protons of quinoline and 4 Ar-H),4.89(2H,s,OCH_2), 3.85 (3H,s,OCH_3)2.83 (3H,s,CH_3).

4-[5-(2-methyl-quinolin-8-yl methoxy)-[1,3,4]oxadiazol-2-yl]-phenol(4a):

IR(Cm^{-1}):3370.64 (OH),3082.6(CH),1611.87(C=N),1166.32(C-O-C),

^{1}HNMR((δPPM): 10.56 (1H,s,OH), 8.15-7.28 (9H,m, C-3,C-4,C-5,C-6,C-7 protons of quinoiline and 4Ar-H), 5.69(2H,s,OCH_2), 2.72(3H,s,CH_3), Mass(m/z): M+ -351.

2-(4-methoxy phenyl)-5-(2-methyl quinolin-8yl) methoxy)-[1,3,4]-oxadiazole(4h):

IR(Cm^{-1}):3083.1(CH),1608.2(C=N),1165.3(C-O-C),

^{1}HNMR(δ PPM): 8.62-6.98(9H,m, C-3,C-4, C-5,C-6, C-7 and 4 Ar-H), 5.36(2H,s,OCH_2), 3.96(3H,s,OCH_3) 2.69(3H,s,CH_3) Mass,m/z – M^+- 334.

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Phytochemical screening and analgesic activity of methanolic extract of *Ximenia americana*

SIDDAIAH. M*[1], JAYAVEERA. K.N[2], MALLIKARJUNA RAO. P[3]
YASODHA KRISHNA Y[4], NARENDER REDDY, G[5]
[1] Annamacharya College of Pharmacy, Rajampet, Kadapa (Dist), Andhra Pradesh, India
[2]Department of Chemistry Jawaharlal Nehru Technological University College of Engineering, Ananthapur, Andhra Pradesh, India.
[3]International Medical University Sesama Center, Plaza Komanwel, Kualampur, Malysia – 57000.
[4] P. Rami Reddy College of Pharmacy, Kadapa, Andhra Pradesh, India.
[5] Senior Lecturer, Government Polytechnic, Suryapet, Andhra Pradesh, India.

ABSTRACT

The analgesic activity of the methanol extract of *Xeminia Americana* leaf extract (MEXA) was investigated in chemical models of nociception in mice. MEXA at doses of 200, 400 and 600mg/kg i.p. produced an inhibition of 54.13, 63.74, and 66.4% respectively, of the abdominal writhes induced by acetic acid in mice. In the formalin test, the administration of 200, 400 and 600mg/kg i.p. had no effects in the first phase (0 to 5 min) but produced a dose dependent analgesic effect on the second phase (15 to 40 min) with inhibitions of the licking time of 29.3, 47.8 and 59.8%, respectively. These observations suggest that MEXA possesses analgesic activity.

Key words: *Xeminia Americana,* acetic acid, aspirin, MEXA, analgesic activity.

Introduction

Xeminia Americana is a small tree or shrub which grows widely in the topical and temperature reasons in the world and belongs to family Olacaceae. It is commonly known as false sandal wood, number if plants, which are known to posses Medicines properties, have been in use in the folklore medicine. According to tribal literature available, the *Xeminia Americana* leaves and twigs are used in treating fever and cold. The roots are used in skin problems, headache sleeping sickness, edema, dysentery, mouth ulcers and as a antidote in poison. In northern parts of Nigeria this plant is used for treating malaria [1]. The fruits are used in habitual medicine and in diabetic [2]. The aqueous plant material extract is also used in the treatment of cancer in African Traditional Medicine [3]. The oral evidence indicates that the plant is effective in these conditions but there is no documented scientific evidence to support such use. Hence, the present work was under taken to evaluate the analgesic property and phytochemical screening of methanolic extract of leaves of *Xeminia Americana*.

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**Corresponding Author:*
siddaiah.m@rediffmail.com

Materials and Methods

Plant material

The leaves *Xeminia Americana* collected from the Nallamala forest of Chintala Village Dornala Mandal Ongole District Andhra Pradesh in the month of October 2007 and were taxonomically identified by Botanist of S.V. University and a specimen voucher No.1295 is kept for future reference at S.V. University.

Preparation of extract

The collected leaves were shade dried coarsely powdered by using mechanical grinder and stored in airtight container. The powder was extracted with methanol using soxhlet apparatus. The solvent was then removed under reduced pressure. It was further concentrated and dried in the desiccators for further studies. The percentage yield of the extract is 13%

Phytochemical screening

A preliminary phytochemical screening of methanol extract was carried out as described by Khandelwal K.L [4].

Animals used

Mature albino mice (20-25g) were used for the present

study. The animals were kept in constant temperature (22±2°C), humidity (55%) and light-dark conditions (12/12 light/dark) and provided with standard pellet diet (Hindustan Lever) and water *ad libitum.* The experiments were performed under the guidance of Ethical committee of Annamacharya College of Pharmacy (Registration No: 1220/a/CPCSE/ACP).

Analgesic activity

Inhibition of acetic acid-induced writhing in mice

Male albino mice were used in groups of six animals per dose of drugs. The animals were pretreated with methanolic extract of *Xeminia Americana* (MEXA) (200, 400 and 600 mg/kg i.p. or aspirin 100 mg/kg, i.p.) for 30 min or 20 min, respectively, prior to intra peritoneal injection of 1% acetic acid (0.1 ml/10 g). Five minutes after the *i.p* injection of acetic acid, the number of writhes was counted for ten minutes. Control mice received normal saline. The responses of extract treated groups were compared with those of animals receiving aspirin 100mg/kg (as standard drug), as well as with the controls [5].

Formalin test

Male Albino mice were used in groups of six animals per dose of drugs Male. Each animal was tested once only. The MEXA (200,400 and 600 mg/kg .) and aspirin (100 mg/kg) were suspended in Tween 80 plus 0.9% (w/v) saline solution and administered *i.p,* in a volume of 0.2 ml. Control group received only drugless vehicle (0.2 ml).. One hour before testing, the animal was placed in a standard cage that served as an observation chamber. 20µl of 1.0% formalin injected to the dorsal surface of the right hind-paw. The mice were observed for 40 min after the injection of formalin, and the amount of time spent licking the injected hind-paw was recorded. The first 5 min post formalin injection is known as the early phase and the period between 15 min and 40 min as the late phase. The drugs were administered 30 min before injection of formalin. The total time spent on licking or biting the injured paw (pain behavior) was measured with a stopped watch. The activity was recorded in 5 min interval [5].

Statistical analysis

Results are presented as mean± SEM. Statistical analysis of data performed using ANOVA followed by Turkey's test.

Results

Preliminary phytochemical studies revealed that the presence of tannins, saponins, alkoloids, glycosides, flavanoids, triterpenoids, sterols and phenolic compounds.

Inhibition of acetic acid-induced writhing in mice

Table-1 shows the pain behavior of writhing response, which is presented as cumulative abdominal stretching response. The treatment of animals with MEXA extract (400 and 600mg/kg. i.p.) produced a significant ($P < 0.01$) and dose dependent inhibition of the control writhes. The inhibition by MEXA (600 mg/kg) was similar to that produced by aspirin (100 mg/kg).

Formalin test

MEXA (400 mg/kg, 600 mg/kg) produced significant ($P < 0.001$) inhibition in the late phase of formalin induced pain; respectively (Table-2). The positive control aspirin (100 mg/kg) also produced significant ($P < 0.001$) inhibition in the late phase.

Discussion and Conclusion

The methanol extract of leaf of *Xeminia Americana* (MEXA) given i.p. at doses of 200, 400 and 600 mg/kg significantly inhibited the acetic acid-induced writhing response in a dose-dependent manner. Acetic acid, which is used as an inducer for writhing syndromes and, causes analgesia by releasing of endogenous substances, which then excites the pain nerve endings; the abdominal constriction is related to the sensitization of nociceptive receptors to prostaglandins [6]. Also, it is well known that, the acetic writhing test is normally used to study the peripheral analgesic effects of drugs. Although this test is a nonspecific (e.g., anti-cholinergic, antihistaminic and other agents also show activity in the test), it is widely used for analgesic screening and involves local cholinergic and histaminic receptors, and the mediators acetylcholine and histamine [7]. This result indicates that the analgesic effect of MEXA might be mediated by its peripheral effect.

The formalin test is a valid and reliable model of nociception and is sensitive for various classes of analgesic drugs. Formalin test produced a distinct biphasic response and different analgesics may act differently in the early and late phases of this test. Therefore, the test can be used to clarify the possible mechanism of antinociceptive effect of a proposed analgesic effect [8]. Centrally acting drugs such as opioids inhibit both phases equally [7], but peripherally acting drugs such as aspirin, indomethacin and dexamethasone only inhibit the late phase. The late phase seems to be an inflammatory response with inflammatory pain that can be inhibited by anti-inflammatory drugs [9, 10]. The effect of MEXA on the late phase of formalin test suggests that its activity may be resulted from its peripheral action when compared with aspirin activity in this respect. Based on the results of this study, we suggest that the analgesic effect of *Xeminia Americana* may be attributed to inhibition of prostaglandin release and other mediators involved in this test. A preliminary phytochemical study reveals that presence of tannins, alkaloids, flavanoids, triterpenoids and sterols. Hence, the present analgesic activity of *Xeminia Americana* may attribute to the presence of tannins, saponins, alkoloids, glycosides, flavanoids, triterpenoids, sterols and phenolic compounds.

In conclusion, this study has demonstrated using pharmacological models of pain that *Xeminia Americana* possesses analgesic effect and this provides a rationale for its use in folk medicine. However, more work is needed to evaluate the mode of action and identify the compounds responsible for its analgesic effect.

Table 1:
Analgesic effect of methanolic extract of *Xeminia Americana* (MEXA) in the acetic acid-induced writhing test[a]

Design of treatment	Number of writhings [a]	Inhibition (%)
Control	75 ± 1.25	-
MEXA (200mg/kg)	34.4 ±2.12***	54.13
MEXA (400mg/kg)	27.2 ± 1.08***	63.73
MEXA (600mg/kg)	25.2 ± 1.27***	66.40
Aspirin (100mg/kg)	28.16 ± 1.17***	62.45

[a] Values are mean±S.E.M.

* $P<0.05$, ** $P<0.01$ *** $P<0.001$ significant compared with control values.

Table 2.
Analgesic effect of methanol extract of *Xeminia Americana* (MEXA) in the formalin test

Design of treatment	Licking(s)[a]		
	0-5min	15-40min	Inhibition (%)
Control	61.7± 3.1	117.7±9.2	-
MEXA (200mg/kg)	59.8±4.4	83.2±7.8*	29.3
MEXA (400mg/kg)	54.9±3.7	61.4±5.4**	47.8
MEXA (600mg/kg)	52.3± 2.4	47.2±5.1***	59.8
Aspirin (100mg/kg)	56.8± 4.2	43.8± 2.9***	62.8

[a] Values are mean±S.E.M.

* $P<0.05$, ** $P<0.01$ *** $P<0.001$ significant compared with control values.

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Invitro antioxidant activity of aqueous extract of *Syzygium alternifolium* seeds

RAMESH BABU K, RAJASEKHAR MD, VINAY KUMAR K, SAMPATH KUMAR MR, SAMEENA FATIMA SK AND APPA RAO CH*
Department of Biochemistry, Sri Venkateswara University,
Tirupati-517 502. A.P., India.

ABSTRACT

The present study was designed to evaluate the antioxidant activity of aqueous extract of *Syzygium alternifolium* (SA) seeds. The extract exhibited a dose dependent scavenging activity against 2,2-diphenly-1-picrylhydrazyl (DPPH) radicals, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals, Nitric oxide radicals and Hydrogen peroxide. The antioxidant activity of the SA seed aqueous extract was found to be higher than that of butylated hydroxyl toluene (BHT), a commercially used antioxidant. Further, the extract had relatively higher reducing power, compared to that of ascorbic acid. The total phenolic content of the extract was found to be 149mg/gm. TLC of the aqueous extract using the DPPH as a spraying reagent revealed a yellow spot against purple background indicated the presence of potent antioxidant compounds. In conclusion, *Syzygium alternifolium* seeds possess antioxidant properties and it could be due to the presence of phenolic compounds.

Keywords: Antioxidant activity, 2,2-diphenly-1-picrylhydrazyl (DPPH) radical, 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical, Nitric oxide, Hydrogen peroxide, Reducing power.

Introduction

Oxidation was essential to many living organisms for the production of energy to fuel biological processes. However, oxygen–centered free radicals and other reactive oxygen species (ROS), which were continuously, produced *in vivo*, result in cell death and tissue damage [1]. ROS play an important role in some pathogenesis of serious diseases, such as neurodegenerative disorders, cancer, liver cirrhosis, cardiovascular diseases, atherosclerosis, cataracts, diabetes and inflammation [2]. Compounds that can scavenge free radicals have great potential in ameliorating these diseases [3]. In this respect, flavonoids and other polyphenolic compounds have received the greatest attention [4].

There was an increasing interest in natural antioxidants, e.g., polyphenols, present in medicinal and dietary plants, which might help to prevent oxidative damage [5]. Polyphenols possess ideal structural chemistry for free radical scavenging activity, and they have been shown to be more effective antioxidants *in vitro* than tocopherols and ascorbate. Plants were a major source of phenolic compounds, which were synthesized as secondary metabolites during normal development in response to stress conditions, such as wounding and UV radiation among others [6, 7]. There has been an increasing interest in the use of natural antioxidants, such as tocopherols, flavonoids and rosemary (*Rosmarinus officinalis L.)* extracts for the preservation of food materials in recent years [8], because these natural antioxidants avoid the toxicity problems which may arise from the use of synthetic antioxidants, such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and propyl gallate (PG) [9, 10].

The oxidative damage might be prevented or limited by dietary antioxidants [11]. The most extensively used synthetic antioxidants, such as butylated hydroxyl anisole (BHA), and butylated hydroxyl toluene (BHT), have restricted use in food and have been suspected of being responsible for liver damage and carcinogenesis [12, 13, 14]. Epidemiological and *in vitro* studies strongly suggest that food containing phytochemicals with antioxidants have potentially protective effects against many diseases, including cancer, diabetes and cardiovascular diseases [15].

**Corresponding Author*
E-mail address: chippadar@yahoo.com

Tirumala Hills (Rayalaseema region, Andhra Pradesh, India), which lie geographically in the South Eastern Ghats are known for the rich heritage of flora. A number of plants which are known to possess medicinal properties have been in use in the folkore medicine [16]. One such plant is *Syzygium alternifolium* (wt) walp. *Syzygium alternifolium* belongs to the family Myrtaceae. It is locally known as Mogi or Adavinerudu. Earlier work in our laboratory has shown that aqueous extract of SA seeds have significant antihyperglycemic activity [17]. It is also used for fevers and skin diseases [18]. Other species of this genus *S.cumini* L. Skeel is well known for its antidiabetic and antioxidant activities (19). Bark, leaves and seeds of *S.cumini* are astringent. Its berry as a whole is astringent. Juices of these fruits are stomachic, astringent, diuretic and antidiabetic [20]. Treatment strategies that focus on decreasing oxidative stress as well as enhancing antioxidant defense systems might present important options for treatment of diabetic complications. Hence compounds with both antihyperglycemic and antioxidative properties would be useful antidiabetic agents [21].

There are no reports on the antioxidant activities of *Syzygium alternifolium* (wt) walp.seed. The present study was undertaken to scientifically investigate the invitro antioxidant potentiality of the aqueous extract of *Syzygium alternifolium* (wt) walp.seeds.

Materials and Methods

Collection of plant material

Seeds of *Syzygium alternifolium* (wt.) Walp. were collected from surrounding areas of Tirupati and identified by the Botanist, Department of Botany, S.V.University, Tirupati. A voucher specimen (Herbarium Accession No.121) was deposited in the herbarium, Department of Botany, S.V.University, Tirupati. These seeds were shade dried and powdered.

Preparation of aqueous extract

To prepare aqueous extract the plant seed powder (200 gms) was soaked in distilled water in a glass jar for 2 days at room temperature and the solvent was filtered. This was repeated three to four times until the filtrate gave no coloration. The filtrate was distilled, concentrated under reduced pressure in the Buchi rotavapour R-200 and finally freeze dried. The yield of the extract was 21% (w/w). The extract was preserved in a refrigerator till further use.

***In vitro* Antioxidant assays:** The following methods were used to evaluate antioxidant activity.

DPPH radical scavenging activity

The hydrogen atom or electron donation ability of the extract was measured from the bleaching of the purple-colored methanol solution of 2, 2-diphenly-1-picrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent [22, 23]. 1ml of various concentrations of the extract (25, 50, 75, 100 and 250µg/ml) in methanol were added to 4ml of 0.004 %(w/v) methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against blank at 517nm. The percent of Inhibition (I %) of free radical production from DPPH was calculated by using the following equation

$$I\ \% = [(A\ control - A\ sample) / A\ blank] \times 100$$

Where A control is the absorbance of the control reaction (containing all reagents except the test compound) and A sample is the absorbance of the test compound. Tests were carried out in triplicate.

ABTS radical scavenging activity

The antioxidant activity of aqueous extract was determined with stable $ABTS^+$ cation radical by Re et al method with slight modifications [24]. $ABTS^+$ was produced by reacting 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diamoniumsalt (ABTS) (Sigma chemicals, USA) with potassium per sulfate ($K_2S_2O_8$). Stock solution of ABTS (2 mM) was prepared by dissolving in 50 ml of phosphate buffered saline (PBS), constituting 8.18 g NaCl, 0.27 g KH_2PO_4, 3.58 g $NaHPO_4$.11 H_2O and 0.15 g KCl in 1L of distilled water. The pH of the solution was adjusted to 7.4 with 0.1 M NaOH. $ABTS^+$ was produced by reacting 50 ml of stock solution with 200 ml of 70 mM $K_2S_2O_8$ solution. The mixture was left to stand in the dark at room temperature for 15–16 hours before use. For the evaluation of antioxidant activity, the $ABTS^+$ solution was diluted with PBS to obtain the absorbancy of 0.800 ± 0.030 at 734 nm. Different concentrations (50, 75,100 and 250µg/ml) of the extract was prepared and mixed with 3ml of $ABTS^+$ solution. The absorbance was read at room temperature after 10 minutes at 734 nm. PBS solution was used as a blank. The percent of Inhibition (I %) of free radical production from ABTS was calculated by using the following equation

$$I\ \% = [(A\ control - A\ sample) / A\ blank] \times 100$$

Where A control is the absorbance of the control reaction (containing all reagents except the test compound) and A sample is the absorbance of the test compound. The measurement was performed in triplicate.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was measured by slightly modified methods of Green et al [25] and Marcocci et al [26]. Nitric oxide radicals (N0) were generated from sodium nitroprusside. 1ml of sodium nitroprusside (10mM) and 1.5 ml of phosphate buffer saline (0.2M, pH7.4) were added to different concentrations (25,50,75 and 100µg/ml) of the extract and incubated for 150 min at 25ºC. After incubation 1ml of the reaction mixture was treated with 1ml of griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% napthylehylenediamine dihydrochloride).The absorbance of the chromatophore was measured at 546nm. Butylated

hydroxyl toluene was used as a standard. Nitric oxide scavenging activity was calculated by using the following equation

% of scavenging =

[(A control – A sample) / A blank] x 100

Where A control is the absorbance of the control reaction (containing all reagents except the test compound) and A sample is the absorbance of the test compound. Tests were carried out in triplicate.

H_2O_2 scavenging activity

The H_2O_2 scavenging ability of the extract was determined according to the method of Ruch et al [27]. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). 10, 25, 50, 75 &100 µg/ml concentrations of the extract in 3.4 ml phosphate buffer were added to H2O2 solution (0.6mL, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. The percent of scavenging of H_2O_2 was calculated by using the following equation

% of scavenging = [(A control – A sample) / A blank] x 100

Where A control is the absorbance of the control reaction (containing all reagents except the test compound) and A sample is the absorbance of the test compound. Tests were carried out in triplicate.

Reducing Power

The reducing power was determined according to the method of Oyaizu [28]. Different concentrations of the extract (25, 50, 250 and 500µg/ml) prepared in methanol were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5ml, 1%). The mixture was incubated at 50°C for 20 min and 2.5ml of trichloroaceticacid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 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 was measured at 700nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a standard.

Determination of total phenolic compounds

Total phenolic content of the extract was determined by Folin-Ciocalteau reagent according to Singleton and Rossi [29] using gallic acid as a standard. 0.1ml (100µg) of sample solution was made up to 3 ml with distilled water. About 0.5 ml of Folin-Ciocalteau reagent was added and mixed thoroughly. Incubated for 3 min at room temperature. After incubation 3ml of 20% Na_2CO_3 was added and mixed thoroughly, incubated in boiling water bath for 1min. The absorbance was measured at 650nm.The concentration of total phenols was expressed in terms of micrograms of gallic acid equivalents.

TLC-DPPH antioxidant screening

This method is generally used for the screening of potential antioxidant activity in crude extracts. It involves the chromatographic separation of the crude plant extract, after which the developed chromatogram is sprayed with a colored radical solution and the presence of antioxidant compounds indicated by the disappearance of radical color. 10µl of the extract was loaded as a 1cm band on the origin of TLC (Merck, Silica gel 60 F_{254} plates). Plate was developed using the methanol: acetonitrile (7:3) solvent. To detect antioxidant activity, chromatogram was sprayed with 0.2% DPPH in methanol as an indicator [30] until just wet and dried in fume hood. The presence of antioxidant compounds were detected as yellow spots against a purple background on TLC plates sprayed with 0.2% DPPH in methanol.

Results and Discussion

The participation of reactive oxygen species in the etiology and pathophysiology of human diseases such as neurodegenerative disorders, inflammation, viral infection, autoimmune pathologies and digestive system disorders such as gastrointestinal inflammation and gastric ulcers was already evident. To understand the role of these reactive oxygen species in several disorders and potential antioxidant protective effect of natural compounds on affected tissues were topics of high current interest. Initially it was necessary to investigate *in vitro* antioxidant properties of any natural product or drug to consider it as an antioxidant substance, followed by evaluation of its antioxidant function in biological systems.

Phytochemical analysis revealed the presence of phenols, flavonoids, carbohydrates and proteins in the aqueous extract of seeds of *Syzygium alternifolium.*

DPPH is a stable nitrogen-centered free radical, and its color changes from purple to yellow when reduced by either hydrogen- or elctrondonation. Substances to perform this above reaction can be considered as antioxidants and therefore radical scavengers [31]. The DPPH radical scavenging activity was known to correlate well with the inhibitory capacity of lipid peroxidation of a test compound [32]. In the present study, it was observed (Figure.1) that purple color of DPPH was bleached completely and very rapidly by the seed aqueous extract at all concentrations (25-250 µg/ml) in a concentration dependent manner. The maximum free radical (DPPH) scavenging activity was exerted by the extract (93%) at a concentration of 250 µg/ml, it was greater than that of standard BHT (67%) at the same concentration.

The aqueous extract had almost equal potent activity to the standard BHT in scavenging $ABTS^+$ radicals. Figure 2 demonstrated a steady increase in the percentage inhibition of the $ABTS^+$ radicals at all concentrations (50-250 µg/mL) in a concentration dependent manner by the

Figure 1
Scavenging effect of *Syzygium alternifolium* aqueous *extract* and standard BHT on 2, 2'-Diphenyl-1-picryl hydrazyl (DPPH) radical. Results are mean ± S.E of three parallel measurements.

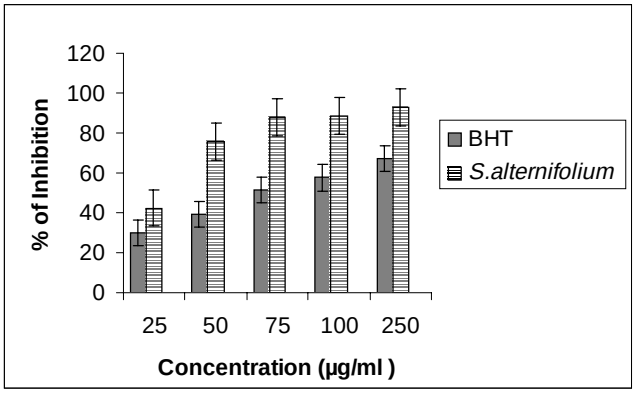


Figure 2
Scavenging effect of *Syzygium alternifolium* aqueous extract and standard BHT on $ABTS^{+}$ radical. Results are mean ± S.E of three parallel measurements

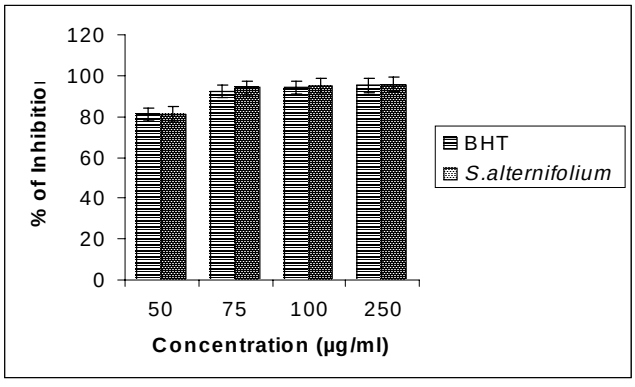


Figure 3
Scavenging effect of *Syzygium alternifolium* aqueous extract and standard BHT on nitric oxide radical. Results are mean ± S.E of three parallel measurements

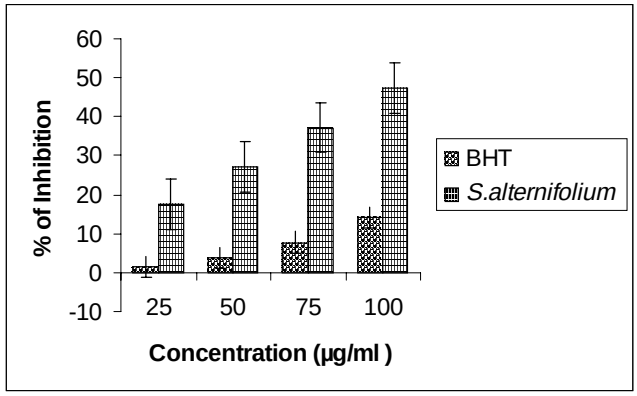
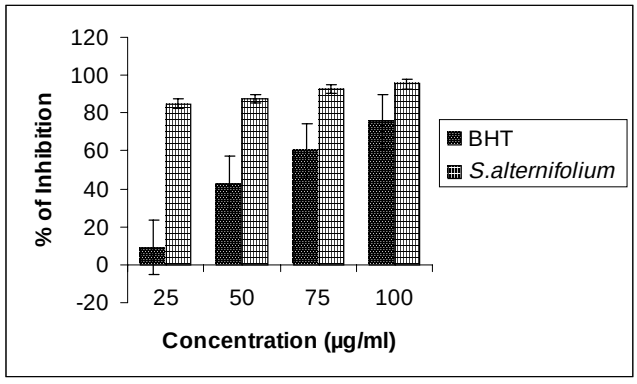


Figure 4
Scavenging effect of *Syzygium alternifolium* aqueous extract and standard BHT on hydrogen peroxide. Results are mean ± S.E of three parallel measurements



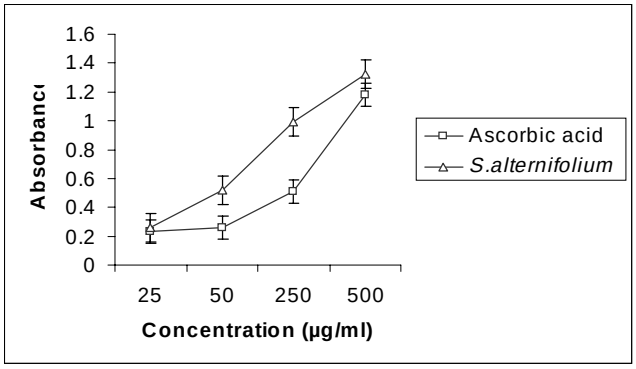
aqueous extract of *Syzygium alternifolium* seeds and maximum inhibition (96%) was achieved at 250 µg/mL of the dry extract. This was slightly higher than the standard BHT (95%) at the same concentration.

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Overall, the aqueous extract of *Syzygium alternifolium* had a better nitric oxide scavenging ability compared to the standard BHT (Figure 3). Aqueous extract at a concentration of 100 µg/ml, inhibited almost 47.5 % of nitric oxide where as BHT inhibited only 14% at the same concentration.

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects [33]. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. Hydroxyl radical is the most reactive among reactive oxygen species (ROS) and it bears the shortest half-life compared with other ROS, induces severe damage to adjacent biomolecules [34]. The scavenging ability of aqueous extract of *Syzygium alternifolium* on hydrogen peroxide was shown in Figure 4. Hydrogen peroxide scavenging activity was increased with increasing concentration of the extract (25-100µg/mL). Aqueous extract at a concentration of 100 µg/ml had maximum activity (95%) than the standard BHT (76%) at a same concentration.

The reducing power has been used as one of the important antioxidant capabilities for medicinal herbs [35, 36]. The reducing power of the aqueous extract of *Syzygium alternifolium* was dose-dependent (Figure 5). The maximum activity was exerted by extract at a concentration of 500 µg/

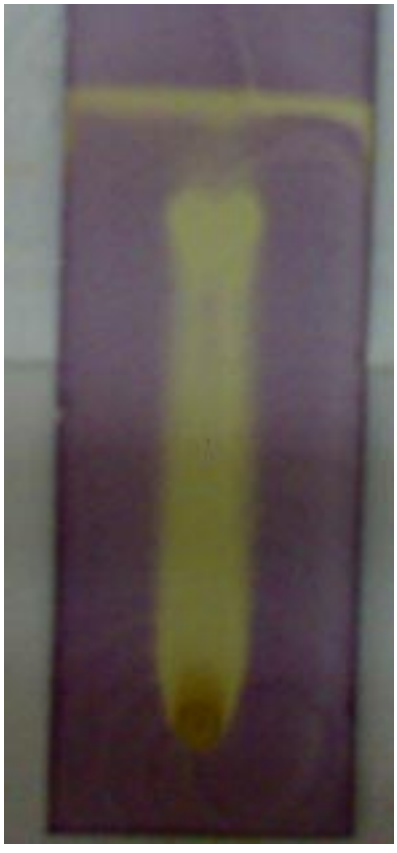
Figure 5 t
The reductive ability of *Syzygium alternifolium* aqueous extract and Ascorbic acid. Results are mean ± S.E of three parallel measurements



ml, this activity was higher than that of standard Ascorbic acid at the same concentration.

The amount of total phenolics present in aqueous extract was 149 mg/g of dry extract. Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the total antioxidant activity of many fruits and vegetables [37]. We have generally found correlation between antioxidant activity and total phenolic content.

Figure 6
TLC –DPPH Chromatogram



The TLC-DPPH chromatogram reveals the presence of potent antioxidant compounds, which were detected as yellow spots against a purple background on TLC plate sprayed with 0.2% DPPH in methanol. The Chromatogram developed with the aqueous extract of the seeds of *Syzygium alternifolium* (figure 6) showed a large yellow spot against purple background on TLC plate, confirming the presence of potent antioxidant compounds in the *Syzygium alternifolium* seeds.

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