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VIEWS

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Phytochemical, Antioxidant and Antimicrobial Studies on Ethanolic and Methanolic Extracts of *Aerva Tomentosa Linn*

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ABSTRACT

The present study was designed for the investigation of phytochemical, antioxidant and antimicrobial activity on ethanolic and methanolic extracts of whole plant of *Aerva tomentosa. Linn.,* belongs to family Amaranthace, which is widely used in ayurveda to cure many remedies. Phytochemical investigation showed the presence of tannins, carbohydrate, glycosides. Antioxidant screening was studied by using H_2O_2 scavenging, reducing power and phosphor molybdenum method. Antioxidant studies reveal that both extracts of *Aerva tomentosa Linn.* showed highest antioxidant activity. Antimicrobial activity was studied by cup-plate agar diffusion method against streptomyceas gresius, bacillus subtilis, corenebacterium, staphylococcus aureus, staphylococcus epidermidis, Escherichia coli, and proteus vulgaris and salmonella typhi by using erythromycin and tetracycline as standard. The results of the study revealed that the ethanolic and methanolic extracts exhibit antimicrobial activity against both gram positive and gram negative bacteria's.

Keywords: *Aerva tomentosa. Linn.,* phytochemical, antioxidant, antimicrobial studies.

Introduction

Aerva tomentosa. Linn. belongs to the amaranthace family, Literature reveals that the various species belonging to amaranthace family traditional used in ayurveda as a medicinal as well as food supplements. *Aerva tomentosa. Linn.* is a perennial wild plant which grows in pasture lands and hills. Traditionally it is used in the treatment of Yoke gall is a common problem in bullock sand is treated with extracts of a perennial plant locally called 'safedbuvariyo' (Aerva tomentosa), its is also been reported by traditional healers for the treatment of laxative, anti helmintics, constipation, skin diseases, liver and antiviral. Studies on chemical and pharmacological activity [1], diuretic activity on ethanolic extract of *Aerva tomentosa. Linn [2].*

Antioxidants are free radical quenching agents and used for the prevention of many diseases. Polyphenolic compounds are commonly found in both edible and non-edible plants and reported to have multiple biological effects due to their antioxidant activity. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents [3]. Free radicals are fundamentals to any biochemical process bond represent an essential part of aerobic life and metabolism. They are continuously produced by the body during respiration and some cell–mediated immune functions. These free radicals are also generated from environmental pollutants. Cigarette smoke automobile exhaust fumes, radiation and pesticides. Free radicals when accumulated in cells cause cumulative damage of proteins, lipids, DNA, carbohydrates and membranes, resulting in oxidative stress. Oxidative stress causes food deterioration, aging and a wide range of human diseases including Alzheimer's disease, Parkinson's disease ,dispatches, cancer diseases .Recently; interest has considerably increased in identifying naturally occurring antioxidants to replace synthetic antioxidants or they cause toxin side effects such as cancers, many antioxidant compounds derived from plants have been identified as free radical (or) active oxygen scavengers. Therefore, plant –derived antioxidants are now receiving a special attention. The present study is designed to investigate phytochemical, antioxidant and antibacterial activity of the ethanolic and methanolic extracts of whole plant of *Aerva tomentosa. Linn.*

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Materials and Methods

Collection and authentication

Aerva tomentosa. Linn. were collected during the month of October to February from the local area of Anantapur district, Andhra Pradesh, India and authenticated by Dr. Ravi Prasad Rao, Dept. Of Botany, Sri Krishna Devaraya University, Anantapur.

Experimental part:

Preparation of extracts:

The whole plant was shade dried, coarsely powdered by using cutter mill and extract with ethanol and methanol in soxhlet extractor. The extract were concentrated and dried by using rota vapour (Heidalph) at 60^0 C under vacuum. The percentage yield of ethanolic and methanolic extracts of whole plant of *Aerva tomentosa Linn.* were found to be 9 and 8gms respectively.

Preliminary phytochemical screening:

The crude ethanolic and methanolic extracts is dissolved in distilled water and subjected for preliminary phytochemical screening. The study was carried out by using standard procedure d[4], [5]. The phytochemical reports are tabulated in Table.1 respectively.

Scavenging of hydrogen peroxide:

A solution of H_2O_2 (20mm) was prepared in phosphate buffer saline (PBS, PH 7.4). Various concentration (20µg-100µg) of standard and extracts was prepared, 1ml of the extract and standard was dissolved in methanol in a separate volumetric flask and to this solution 2ml of H_2O_2 solution in PBS was added, the absorbance was measured at 230nm, after 10min against blank solution.

Determination of Reducing Power:

Method based on the principle of increase in the absorbance of the reaction mixture. Increase in the absorbance indicates increase in anti-oxidant activity [6]. Different concentration of extracts (20µg-100µg) in 1ml of distilled water were mixed with 2.5ml of phosphate buffer (0.2m;P--H6.6) & 2.5ml of potassium ferricyanide [K3Fe(CN)6] (1%), the resulting mixture was incubated at 50^oC for half an hour. Then, 2.5ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000rpm for 10min. Finally 2.5ml of upper layer solution was mixed with 2.5ml of distilled water and 0.5ml of $FeCl_3$ (0.1%) were added. The absorbance was measured at 700nm in UV-Vis spectrophotometer against blank. Increasing of the reaction mixture indicates increasing reducing power [7].

Estimation of Phosphomolybdenum:

In this method quantitative determination of anti-oxidant capacity, through the formation of phosphor molybdenum complex. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate Mo (V) complex at acidic pH. An aliquot of 0.3ml of sample solution containing a reducing species in DMSO was combined in a test tube with 3ml of reagent solution (0.6m H_2SO_4, 28mm sodium phosphate and 4mm ammonium molybdate) then the tubes were covered with aluminium foil and kept in a water bath at 95^Oc for 90min. Then the samples were cooled to room temperature, absorbance of each solution was measured at 695nm against blank. The total anti-oxidant was expressed as mm equivalent to ascorbic acid [8]. The results are tabulated in (Table 2) respectively.

Anti microbial activity by ager diffusion method:

The anti microbial activity was evaluated by employing overnight cultures kept for 24 hrs at 36^0C of Streptomyces gresius, Bacillus subtillis, Coryne bacterium, Staphylococus aureus, Staphylococus epidermidies, E.Coli, Proteus vulgaris, Salmonella typhi were prepared using different mediums. The plates were prepared with agar medium left at room temperature for solidification. Accurately different dilutions of methanolic and ethanolic extracts of *Aerva tomentosa Linn.* (250µg, 500µg, 750µg and 1000 µg) and standard antibiotics (erythromycin and tetracycline) solutions were transferred to wells aseptically and labelled accordingly. The plates were incubated at 37^0 C for 24 hours. The diameter of zones inhibition surrounding each of wells was recorded and tabulated in Table 3 [8-10].

Results and Discussions

Phytochemical screening of methanolic and ethanolic extracts of *Aerva tomentosa. Linn* revels the presence of tannins, carbohydrates, glycosides as majorly compounds tabulated in Table no 1. Antioxidant studies prove to show potent antioxidant activity for both methanolic and ethanolic extracts of Aerva *tomentosa. Linn*. Presence of the tannins in past reported to possess antioxidant properties [12]. Hydrogen peroxide scavenging methanol extract showed high activity than ethanol extract, the reducing anti-oxidant activity shows the reducing property of the plant extracts on potassium ferricyanide. The absorbance is directly proportional to the reduction of ferric ions to ferrous ions, thus an increase in the absorbance denotes the reducing property of the plant extracts, methanol extract show higher anti-oxidant activity than ethanol extracts. In Phosphomolybdenum method the two extracts show similar activity. Microbial study of methanol and ethanol extracts showed high zone of inhabitation than the standard antibiotics (erythromycin and tetracycline).

Table-1:
Preliminary Phytochemical Screening Test

Chemical Tests	Methanol extract	Ethanol extract
Test for Carbohydrates:		
1. Molisch's Test		
2. Fehling's Test	—	-ve
3. Barfoed's Test	+ve	-ve
4. Benedict's Test	—	-ve
5. Seliwanoff's Test	+ve	+ve
6. Pentose's Test	-ve	-ve
7. Keller-Kiliani Test	-ve	-ve
	+ve	+ve
Test for proteins:		
1. Biuret Test		
2. Xanthoproteic Test	-ve	-ve
3. Ninhydrin Test	-ve	+ve
4. Nitroprusside Test	-ve	-ve
5. Lead-Sulphide test	-ve	+ve
	+ve	+ve
Test for Flavonoids:		
1. Sinoda Test		
2. Lead-Acetate Test	-ve	-ve
3. Sodium hydroxide test	-ve	-ve
	-ve	+ve
Tests for Fixed oils:		
1. Boundin's Test		
2. Persic oil Test	-ve	-ve
-ve	+ve	
Tests for Volatile oils:		
1. Test I		
2. Test II	+ve	-ve
	-ve	-ve
Test for Tannins:		
1. Gelatin Test		
2. Catechin Test	-ve	+ve
3. Chlorogenic acid Test	+ve	+ve
4. Vanillin-HCl Test	+ve	+ve
5. Gombir-Flurescin Test	-ve	-ve
6. Ferricchloride Test	+ve	+ve
	-ve	+ve
Test for Resins:		
1. Test I		
2. Test II	-ve	-ve
3. Test III	-ve	-ve
	-ve	-ve
Test for Alkaloids:		
[1] Dragnedroff's Test		
[2] Wagner's Test	+ve	+ve
[3] Hager's Test	-ve	-ve

Contd.,

[4] Van-Urk's Test	-ve	-ve
[5] Talleoquin Test	-ve	-ve
[6] Tannic acid test	-ve	+ve
[7] Vitalimorin test	+ve	+ve
	-ve	-ve
Test for Glycosides:		
1. Keller-Killiani test		
2. Barn-Tager test	+ve	+ve
3. Modified Born-Tager's test	-ve	-ve
4. Saponinn glycosides test	+ve	+ve
5. Baljet's test	-ve	+ve
6. Hydroxy Xanthoquinone's test	+ve	+ve
7. Legal test	-ve	-ve
8. Bromine test	-ve	-ve
9. Klunge's isobarbaloin's test	-ve	+ve
10. Flavanoid glycosides test	-ve	-ve
11. Antimony hydrochloride test	-ve	-ve
12. Tollen's test	-ve	-ve
	—	-ve
Test for Others:		
1. Waxes		
2. Insulin	+ve	-ve
	—	-ve

+ve – presence of phytochemicals and –ve – absence of phytochemicals

Table-2:
Anti-oxidant activities of Methanolic and ethanolic extracts of Aerova tomentosa Linn

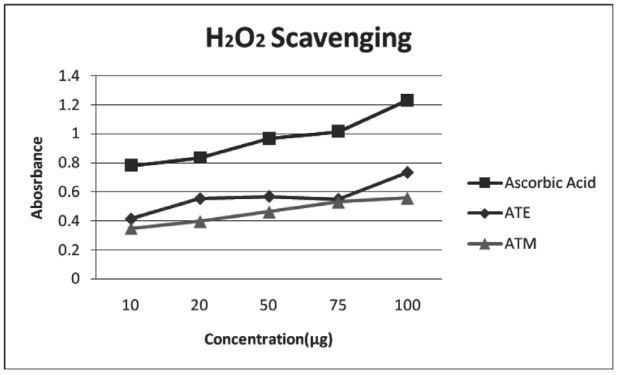
H_2O_2-SCAVENGING		REDUCING POWER		PHOSPHOMOLYBDENUM	
Sample	**Absorbance**	**Sample**	**Absorbance**	**Sample**	**Absorbance**
ASCORBIC ACID	0.780±0.03	ASCORBIC ACID	0.530±0.05	ASCORBIC ACID	0.530±0.04
	0.836±0.03		1.055±0.06		1.055±0.08
	0.968±0.04		1.107±0.09		1.107±0.08
	1.016±0.06		1.792±0.07		1.792±0.09
	1.232±0.08		2.433±0.10		2.433±0.10
ATE	Absorbance	ATE	Absorbance	ATE	Absorbance
	0.415±0.04		0.192±0.02		0.097±0.03
	0.554±0.05		0.173±0.01		0.065±0.02
	0.568±0.03		0.180±0.02		0.084±0.04
	0.550±0.06		0.178±0.03		0.077±0.06
	0.733±0.07		0.182±0.08		0.082±0.07
ATM	Absorbance	ATM	Absorbance	ATM	Absorbance
	0.348±0.03		1.895±0.02		0.091±0.08
	0.396±0.04		2.001±0.09		0.074±0.06
	0.462±0.05		1.821±0.08		0.078±0.07
	0.530±0.05		1.618±0.08		0.102±0.09
	0.557±0.06		1.912±0.09		0.124±0.09

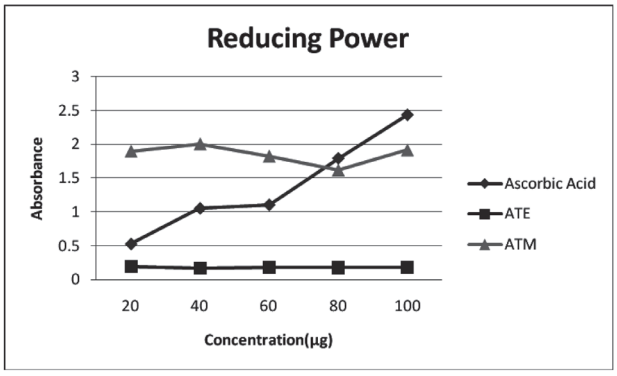
ATE : Ethanol extract of *Aerva tomentosa*

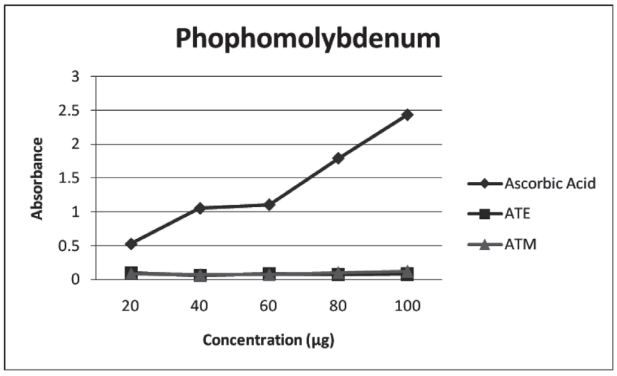
ATM: Methanol extract of *Aerva tomentosa*

Table-3:
Antimicrobial studies of Aerva tomentosa Linn

S.NO	Organisms	Methanol extract				Ethanol extract				Std
		250 µg	500 µg	750 µg	1000 µg	250 µg	500 µg	750 µg	1000 µg	250 µg
1	**Streptomyces gresius**	8.4	11.5	14.2	15.2	14	15.1	16.2	17.1	21
2	**Bacillus subtillis**	13.2	13.8	14.7	16.5	14.1	15.2	16	17.5	27
3	**Coryne bacterium**	9.2	13.2	13.4	14.2	12.1	13.6	15.2	16.3	28
4	**Staphylococus aureus**	9.4	13.4	15.1	17.1	13.8	15.2	17.1	18.2	21
5	**Staphylococus epidermidies**	12.4	15.4	14.3	15.9	9.2	11	12.3	13.4	26.4
6	**E.Coli**	12	13.6	14.5	15.1	11.2	14.2	14.8	15.2	17.2
7	**Proteus vulgaris**	11.3	13.4	15	15.8	13.8	15	16.2	17.2	18.6
8	**Salmonella typhi**	14	16.8	17.6	17.6	14.5	15.1	16	17.2	18.6







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Antibacterial Activity and Free Radical Scavinging Activity of Methanolic Extract of *Kigelia Africana* Flowers

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ABSTRACT

Present study was designed to carry out antibacterial properties of methanolic flower extract of *Kigelia africana* was determined against *Bacillus subtilis*, *Proteus vulgaris*, *Escheria coli,* , *Staphylococcs aureus*, *Pseudomonas aeruginosa* and Tetracycline and erythromycin used as a standard drugs by cup plate agar diffusion method . The minimum inhibitory concentrations were determined using the micro well dilution method. These plates were incubated at 37°C for about 24 to 48hrs. After incubation period zone of inhibition was observed around disc was measured and recorded, the minimum zone of inhibition were susceptibility to *B.subtilis* 50µg/ml*, P.vulgaris* 10 µg/ml, *E.coli* 50 µg/ml, *S.arures* 10 µg/ml, *P.areuginosa* 100 µg/ml respectively. The methanol extract was subjected to preliminary phytochemical analysis. Free radical scavenging activity of methanol extract at different concentration was determined with three methods namely reducing power, H_2O_2 and phospho molybdenum method. Susceptibility of assay was carried out with 20mg / ml concentration, the concentration of the each suspension is 10^6cfu/ml. this was poured in to sterile Petri dishes and used for analysis. The flavonoids and glycosides were detected as phytoconstituents of the methanol flower extracts.

Key words: *Kigelia Africana,* Antibacterial, Antioxidant Activity, Phytoconstituents.

Introduction

Nowadays the prevention of many diseases has been associated with the ingestion of different plants rich in antioxidants it was found out that intake of such a compounds is associated with a lower risk of mortality from different kinds of diseases. The protective effect is due to the presence of several components such as proteins, vitamins, carotenoids, flavonoids, (anthocyanins) and phenolic compounds.

Kigelia africana belongs to the family Bignoniaceous, a native of Africa and is commonly known as sausage tree. It is widely cultivated in Africa; roasted fruits are used to flavored beer and aid fermentation. The tough wood is used for shelving and fruit boxes, and dugout canoes are made from the tree in Botswana and Zimbabwe. Roots are said to yield a bright yellow dye. Traditional remedies prepared from crushed, dried or fresh fruits are used to deal with ulcers, sores and syphilis - the fruit has antibacterial activity. Today, beauty products and skin ointments are prepared from fruit extracts. Fresh fruit cannot be eaten - it is said to be a strong purgative, and causes blisters in the mouth and on the skin. Green fruits are said to be poisonous. In time of scarcity, seeds are roasted and eaten. The present study aims to perform the antibacterial and antioxidant activity of methanolic extract of *Kigelia africana flowers.*

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Materials And Methods

Plant materials:

The flowers of *Kigelia africana* were collected from local area of Anantapur, A.P. India in the month of February to March 2010. The plant was authenticated by Dr. Prasad Rao, Department of Botany, Srikirshna Devaraya University, Anantapur, A.P. India.

Preparation of extraction of extract: -

The flowers were collected and shaded dried, coarsely powdered by using cutter mill and extracted with methanol in Soxhlet extractor. For about 16-18hrs and solution was evaporated and dried by using Rota evaporator (Heidolph) under vacuum. The concentrated extract was stored in photo-resistant container.

Preliminary phyto- chemical screening:

The methanolic flower extract was subjected to phytochemical screening for carbohydrates, tannins, glycosides, saponins, alkaloids, flavonoids, and sterols by using standard procedure. [10-11]

Micro organisms used for the Tests:

The microbial strains were collected from the microbiology department, OTRI, JNTU, Anantapur. The selected strains of bacteria are *Pseudomonas aerugonosa, Proteous vulgaris*, *E.coli, Bacillus subtilis* and reference antibiotics are tetracycline and erythromycin.

The Antimicrobial activity determination:

The antimicrobial activity was determined using agar diffusion method. Inoculate of the test organisms obtained from the source were prepared by growing each pure isolates in nutrient broth for 18hrs at 37°c.The over night broth cultures was matched with land's turbidity standard to give an approximate 10^8cfu/ml.0.2ml was then used to seed a molten Muller Hinton agar medium which has been allowed to cool to 45^0c to obtained approximate10^8cfu/ml. This was poured in to sterile Petri dishes and used for analysis. The susceptibility of assay was carried out with 20mg/ml concentration of each extracts with bacterial suspension of 10^8cfu/ml. this was delivered in to wells (8mm in diameter) bored on to the surface of already seeded millers Hinton agar plates. Commercial discs containing tetracycline 100µg served as positive controls for the antibiotic activity .the plates were incubated at 37^0c for 24hrs. After incubation, zone of inhibition around the wells and the disc were measured and recorded.

Determination of minimum inhibitory concentration:

The minimum inhibitory concentrations (MIC) of the extract against the various organisms were determined using the micro well dilution method. The 96-microwell sterile plates were prepared by dispensing 250µl of inoculated broth and 50µl of plant extract constituted in broth. Tetracycline (sigma-Aldrich, USA) was included as positive control. The plates were incubated at 37^0c for 24hrs. Presence of bacterial growth was determined by the addition of 50µl of P-iodonitrotetrazolium violet (0.2mg/ml).

Determination of Antioxidant Activity:

Invitro antioxidant activity of methanolic extraction of was also tested by using three methods Hydrogen Peroxide method, reducing power method, and phosphate molybdenum method. In all methods the extract concentration ranging from 10-100µg/ml concentration was taken with approximate standards and checked for the antioxidant activity. The absorbance was measured against blank that contains extracts and standard. But with out reagent a control performed. The antioxidant activity was measured by specto photometric method as follows.

Antioxidant activity by H_20_2 method

In this method the reactive molecule O_2 species is stable under physiological pH and temperature in the absence of metal ion. It can be reactive from super anion by super oxide disseminate. It can generate the hydroxyl radical in presence of metal ions and super oxide anions a solution of H_20_2 was prepared in phosphate buffer saline. Various concentrations of 1ml of extract or strand methanol were added to 2ml of H_2O_2 in the absence of sample was measured at 230nm after 10minutes again measured blank.

Reducing power method

This method is based on the principle of increase in the absorbance of the reaction mixture. Increasing the absorbance indicates increase in the antioxidant activity in trichloro acetic acid (TCA) and $FeCl_3$ Witch is measured at 700nm. Increase in the absorbance indicates increase in the reducing power. The reducing power of the plant extract was determined by according to the method [9]., different amount of extracts 20-100 µg in 1ml of distilled water were mixed with 2.5 ml of phosphate buffer (PH6.6) and 2.5ml of potassium ferric cyanide and then the mixture was incubated at 50^0c for 30min. After 2.5ml of TCA 10%was added to the mixture which was centrifuged at 3000rpm for 10min finally 2.5ml of upper layer solution was mixed with 2.5ml of distilled water and 0.5ml of $Fecl_3$ (0.1%) and the absorbance was measured at 700nm in spectrophotometer increasing the reaction mixture indicates increased in the reducing power .

Phosphate molybdenum method

It is a spectroscopic method for the quantitative determination of antioxidant capacity through the formation of phosphomolybdanum complex. The assay is based on the reduction of molybdenum IV to molybdenum V by the same analyte and subsequent formation of green phosphate molybdenum V complex at acidic pH.

An aliquot of 0.3ml of sample solution containing a reducing species in dimethyl sulphoxide was combined in a test be with 3ml of reagent solution (0.6, ml H_2So_4, 28ml sodium phosphate and 4ml ammonium molybdate). Then the tubes are covers with aluminum foil and incubate in water both at 95°c for 90 min. Then the samples were cooled at room temperature and the absorbance of each solution was measured at 695nm against blank sample.

Result

The phytochemical analysis of the methanol extract showed the presence of proteins, glycosides, flavonoids. The phytochemical screening of methanol extract of *Kigelia africana* showed in the table no: 1. the methanol extract of *Kigelia africana* had antimicrobial activity against all the test micro organisms, tetracycline's used as standard. The antimicrobial activity of methanol extract was shown in table no: 2 and it is represented in figure no: 1. the scavenger activity of methanol was carried out by three methods. In each method increase in concentration of the extract gradually increasing the anti oxidant activity as reported in table no: 3, 4, 5 and figure no: 2, 3, 4.

Table No: 1
Preliminary phyto chemical screening of methanolic extract of *Kigelia africana* Flowers

S. No	Phytoconstituents	Result
1.	Alkaloids	-Ve
2.	Tannins	-Ve
3.	Saponins	-Ve
4.	Steroids	-Ve
5.	Volatile oil	-Ve
6.	Flavonoids	+Ve
7.	Phenols	-Ve
8.	Proteins	+Ve
9.	Glycosides	+Ve
10.	Resins	-Ve

+Ve— Positive -Ve— Negative

Conclusion

From the above studies it can be concluded that the methanolic extracts of *Kigelia africana* flowers exhibit significant antibacterial activity against pathogenic bacteria. The inhibited activity is due to the presence of flavonoids and glycoside content. Therefore this *Kigelia africana* flowers may be useful to explore as another source of natural antibiotic. This study reaffirms the ethanomedicinal property of *Kigelia africana*. On the basis of the results obtained in the present study, it is concluded that methanol extraction of *Kigelia africana* flowers has potent antioxidant and free radical scavenging activity in different I*nvitro* models system. Thus, antioxidant potential of extract of flowers of *Kigelia africana* may be attributed to the presence of flavonoids. Therefore, a further investigation is needed to isolate and identify the active compounds present in the plant extract and its efficacy to be evaluated.

Table No: 2
Antibacterial activity of methanolic extract of *Kigelia africana* flowers (Zone of Inhibition in mm*, * n= 3)

S.No	Concentrationµg/ml	*B.subtilis*	*P.vulgaris*	*E.coli*	*S.aureus*	*P.areuginosa*
1.	10	0	5	0	6	0
2.	50	5	9	5	9	0
3.	100	8	12	8	10	4
4.	200	10	17	10	15	9
5.	500	15	20	14	17	13

Table No: 3
Scavenging activity of methanolic extract of *Kigelia africana* Flowers by H_2O_2 method

Sl. No.	Standard	Volume of sample	Concentration of sample	Volume of PBS with H_20_2	Absorbance at 230nm
1	Ascorbic acid	1ml	10µg	2ml	1.517
2		1ml	25 µg	2ml	1.554
3		1m1	50 µg	2ml	1.614
4		1ml	75 µg	2ml	1.623
5		1ml	100 µg	2ml	1.659
1	Methanolic extract of *Kigelia africana*	1ml	10µg	2ml	0.838
2		1ml	25 µg	2ml	0.873
3		1m1	50 µg	2ml	1.121
4		1ml	75 µg	2ml	1.118
5		1ml	100 µg	2ml	1.444

Table No: 4

Reducing power activity of methanolic extract of *Kigelia africana* flowers

Sl. No	Standard/ sample	Volume of sample	Conc. Of sample	Distilled water	Phos phate buffer	Pataciu ferric cyanide	Incubation at 50^0c for about 10 min	TCA	Centrifugation at 3000rpm for 10.min	Upper layerof centrifuge	Distilled water	$Fecl_3$	Absorb ance at 700nm
1.	Ascorbic acid	1ml	20µg	1ml	2.5ml	1ml		2.5ml		2.5ml	2.5ml	0.5ml	1.770
2.		1ml	40 µg	1ml	2.5ml	1ml		2.5ml		2.5ml	2.5ml	0.5ml	2.132
3.		1ml	60 µg	1ml	2.5ml	1ml		2.5ml		2.5ml	2.5ml	0.5ml	4.943
4.		1ml	80 µg	1ml	2.5ml	1ml		2.5ml		2.5ml	2.5ml	0.5ml	4.945
5.		1ml	100 µg	1ml	2.5ml	1ml		2.5ml		2.5ml	2.5ml	0.5ml	4.947
1.	Methanolic extract of *Kigelia africana*	1ml	20µg	1ml	2.5ml	1ml		2.5ml		2.5ml	2.5ml	0.5ml	0.235
2.		1ml	40 µg	1ml	2.5ml	1ml		2.5ml		2.5ml	2.5ml	0.5ml	0.148
3.		1ml	60 µg	1ml	2.5ml	1ml		2.5ml		2.5ml	2.5ml	0.5ml	0.201
4.		1ml	80 µg	1ml	2.5ml	1ml		2.5ml		2.5ml	2.5ml	0.5ml	0.180
5.		1ml	100 µg	1ml	2.5ml	1m		2.5ml		2.5ml	2.5ml	0.5ml	0.199

Table No: 5

Scavenging activity of phospho molybdenum of methanolic extract of *Kigelia africana* flowers.

Sl. No.	Standard	Concentration of sample	Volume of sample		Volume of reagent	Absorbance at 695nm
1	**Ascorbic Acid**	20µg	0.3ml	Incubation at 95°c for 90 min. in a water both and cooled to Room temperature.	3ml	0.126
2		40 µg	0.3ml		3ml	0.0273
3		60 µg	0.3ml		3ml	0.297
4		80 µg	0.3ml		3ml	0.385
5		100 µg	0.3ml		3ml	0.52
1	**MGRI METH**	20µg	0.3ml		3ml	0.032
2		40 µg	0.3ml		3ml	0.037
3		60 µg	0.3ml		3ml	0.039
4		80µg	0.3ml		3ml	0.063
5		100 µg	0.3ml		3ml	0.095

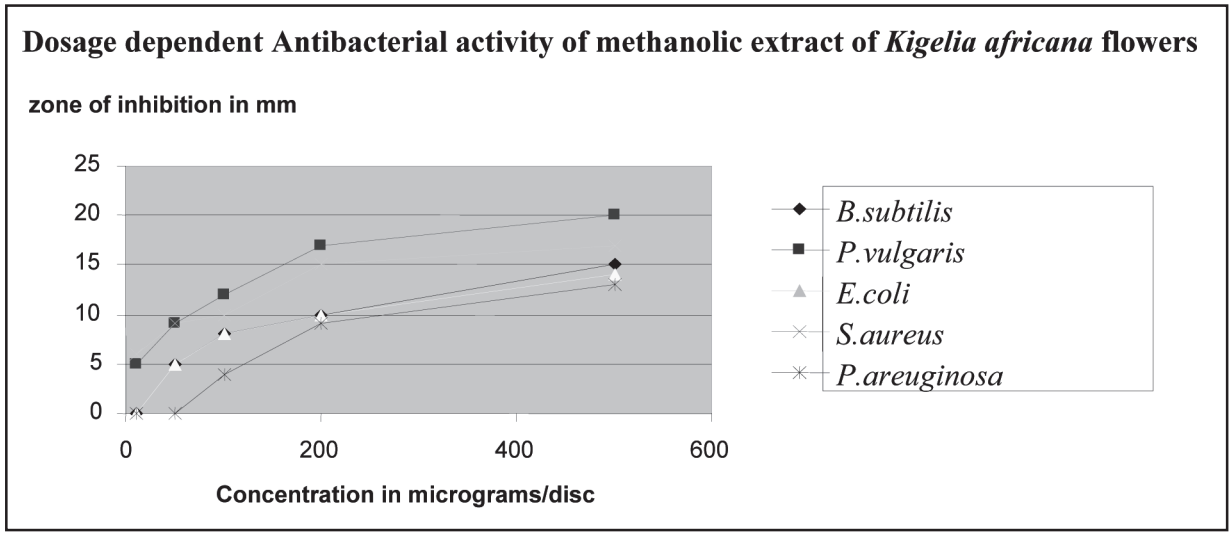

Figure 1

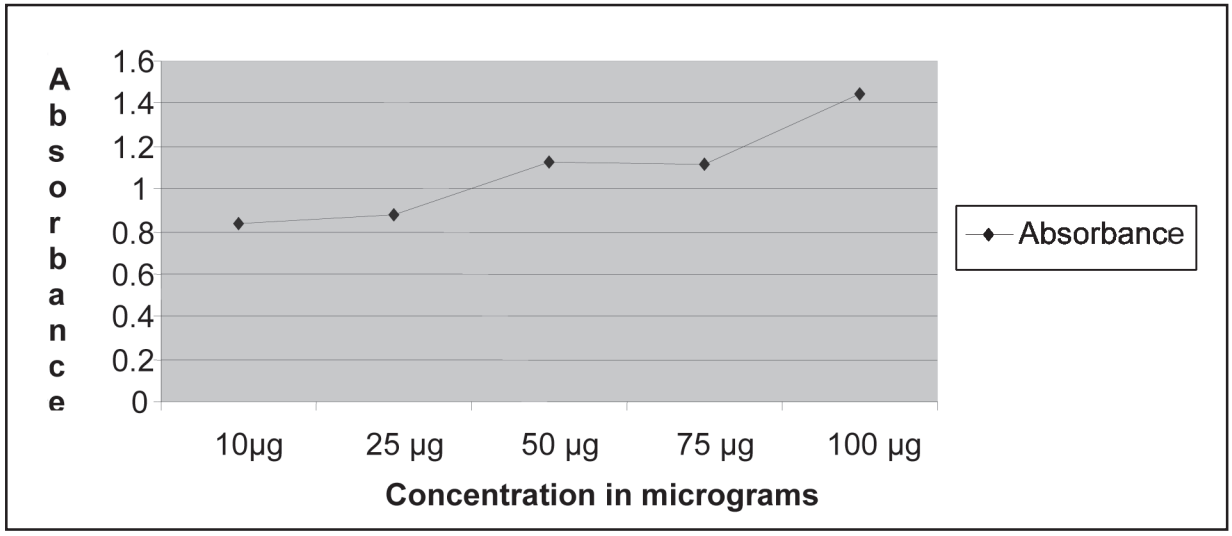

Fig.2: Scavenging activity of methanolic extract of *Kigelia africana* Flowers by H_2O_2 method.

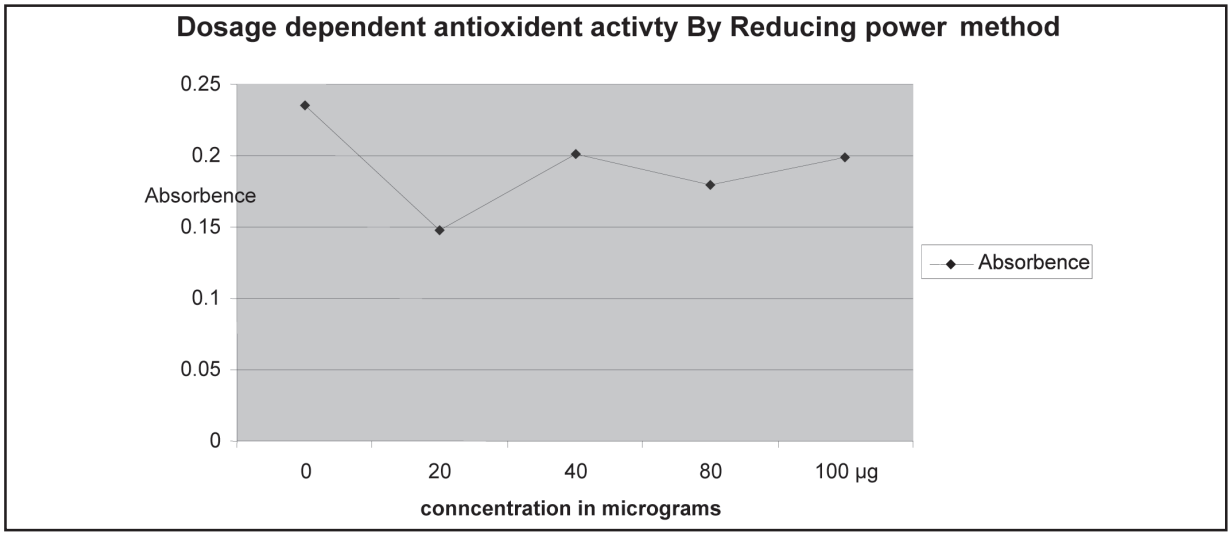

Fig.3: Scavenging activity of methanolic extract of *Kigelia africana* flowers.

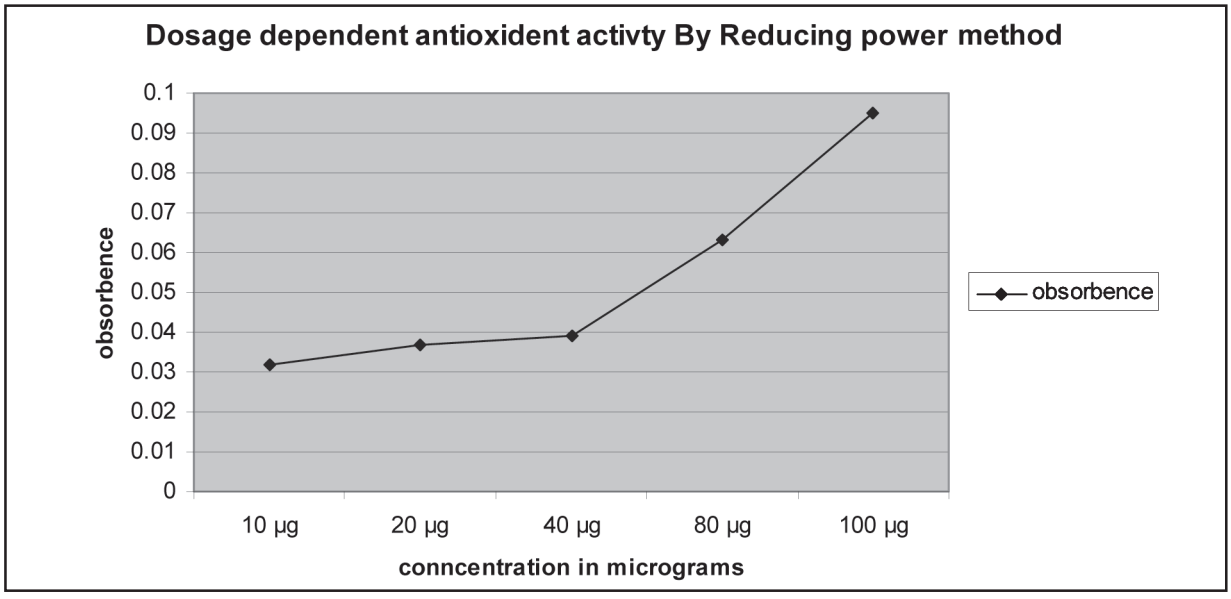

Fig. 4: scavenging activity of methanolic extract of *Kigelia africana* flowers.

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Synthesis of Quinazoline Derivatives as Anti-tubercular Agents

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ABSTRACT

A series of novel (E)-7-chloro-3-substituted-2-phenylquinazolin-4(3H)-one have been synthesized by condensing 3-amino-7-chloro-2-phenylquinazolin-4(3H)-one and added slowly to an ethanolic solution of aromatic carbonyl compound. A mixture of N-chloro anthranilic acid and benzoyl chloride was condensed to form 7-chloro-2-phenyl-4H-benzo[d] [1, 3] oxazin-4-one and various aromatic carbonyl were condensed with ethanolic solution to form. (E)-7-chloro-3-substituted-2-phenylquinazolin-4(3H)-one the structures of the synthesized compounds were elucidated by instrumental data. The synthesized compounds were found to have significant effect against the tested micro organisms and were screened against Mycobacterium tuberculosis using Microplate Alamar blue Assay Method.

Keywords: Synthesis, Quinazolines, Anti tuberculosis

Introduction

Quinazoline nucleus has attracted attention of medicinal chemists, due to wide spectrum of biological activities exhibited by them. Quinazoline and condensed quinazolines have exhibited a variety of biological activities like analgesic, anti-inflammatory[1], anti hypertensive[2], anti histaminic, anticancer[3-5], sedative, hypnotic and antimicrobial activities[6-7]. From the various quinazolines reported the C-2 and N3 disubstituted quinazolines exhibited interacting pharmacological activities. The literature reveals that aromatic carbonyl Schiff base derivative show a wide range of biological spectrum like anticonvulsant, anticancer, antiviral and antimicrobial activities. In spite of large number of quinazoline and aromatic carbonyl have been synthesized and studies have done for various pharmacological activities, however, quinazolinyl aromatic carbonyl derivatives were not reported so far. Hence the present study was, to synthesize some quinazolinyl Schiff derivatives and try to explore their possible biological activities.

Experimental

The melting points were taken in open capillary tubes in concentrated sulphuric acid melting point bath and therefore the values reported are uncorrected. UV spectras were recorded on schimadzu 1700, UV-VIS spectrophotometer and spectral grade ethanol was used as solvent. The IR spectra of the compounds were recorded in the region, 4000-400cm^{-1} using KBr discs on JASCO 4100 FT1R and the NMR spectral study was done using DMSO as solvent on DSX-300/AV-700 transform-NMR spectrometer. Mass spectra studies were done in JEOL GC mate.The purity of the compounds was checked by TLC, using plates coated with silica gel G, Ethyl acetate: Butanol:water as mobile phase and iodine vapour as detection method.

I-Synthesis of 3-amino-7-chloro-2-phenylquinazolin-4(3H)-one [8]

2-Amino-4-chlorobenzoic acid (0.1mol) was dissolved in 30ml of dry pyridine by stirring slowly at room temperature. The solution was cooled to 0°C and a solution of a benzoyl chloride (0.2mole) in 30ml of dry pyridine was added slowly with constant stirring. After this addition the reaction mixture was further stirred for half an hour at room temperature and set aside for 1h. The pasty mass obtained was diluted with 50 ml of water and treated with aqueous sodium bicarbonate solution. When the effervescence ceased, the precipitate obtained was filtered and washed with water. The crude 7-chloro-2-phenyl-4H-benzo[d][1,3]oxazin-4-one obtained was dried and recrystallized from diluted ethanol.

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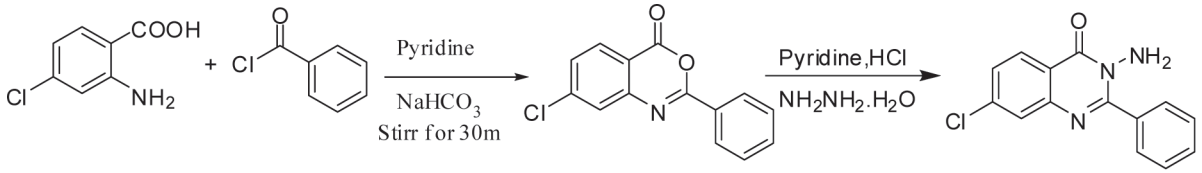


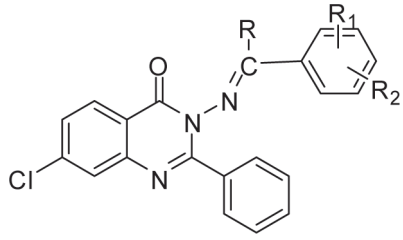
7-chloro-2-phenyl-4*H*-benzo[*d*][1,3]oxazin-4-one

3-amino-7-chloro-2-phenylquinazolin-4(3*H*)-one

To a cold solution of 7-chloro-2-phenyl-4H-benzo[d][1,3] oxazin-4-one (0.05mol) in anhydrous pyridine (20ml) was added a solution of hydrazine hydrate (0.1mol) in anhydrous pyridine (25ml) drop wise with constant stirring. When the addition was complete, the reaction mixture was stirred vigorously for 30min at room temperature and subsequently heated under reflux for 6 h under anhydrous reaction conditions. The reaction mixture was allowed to cool to room temperature and poured into ice cold water containing diluted hydrochloric acid. The obtained crude precipitate of 3-amino-7-chloro-2-phenylquinazolin-4(3H)-one was filtered off, washed repeatedly with water and dried, recrystallized from diluted ethanol.

II-General procedure for the synthesis of titled compounds

3-amino-7-chloro-2-phenylquinazolin-4(3H)-one (0.01mol) was dissolved in ethanol (20ml) and added slowly to an ethanolic solution of aromatic carbonyl compound (0.01mole). The reaction mixture was acidified with 5ml of glacial acetic acid and refluxed for half an hour. The precipitate obtained was filtered and washed with the mixture of ether and water and dried. The product obtained was recrystallized from 95% ethanol



Purification and Spectral studies

Purification of compounds were tested on 5x20cm size plates, coated with silica gel-G to a thickness of 0.25mm.The plates were activated by heating at 110 °C for 1 hour. Benzene: chloroform (80:20) was used as mobile phase with iodine vapour detection. The recrystallized compounds of 10- l00µg/ml concentration were scanned for UV spectra to confirm their λ-max using spectral grade ethanol as solvent. Solid samples (0.5 - 0.lmg) were intimately mixed with appropriate solid samples of dry, powdered potassium bromide. Mixing was effected through a smooth agate mortar and the mixture was pressed between a punch and disc under a pressure of 10,000-15,000psi into a transparent disc. NMR spectral studies were done in ppm concentration of the compounds using DMSO as solvent. Mass spectra studies were done in JEOL GC mate.

1. 7-chloro-3-(4-hydroxybenzylideneamino)-2-phenylquinazolin-4(3H)-one

IR- OH - 3100-3500cm^{-1}, C=O – 1665cm^{-1}, C=N – 1587cm^{-1}

^{1}HNMR- Ar (12H) 7.602 – 8.843δ, OH-10.592 δ, CH=N -8.843 δ

MS – m/z 375.08 (M^+)

2. 7-chloro-3-(2-hydroxybenzylideneamino)-2-phenylquinazolin-4(3H)-one

IR- OH - 2800-3400cm^{-1}, C=O – 1672cm^{-1}, C=N – 1590cm^{-1}

^{1}H NMR- Ar (12H) 7.576 – 8.678δ, OH-8.811δ, CH=N -8.816δ

MS – m/z 375.3051 (M^+)

3. 7-chloro-3-(benzylideneamino)-2-phenylquinazolin-4(3H)-one

IR- C=O – 1660cm^{-1}, C=N – 1574cm^{-1}

^{1}H NMR- Ar (13H) 7.5 – 8.813δ, CH=N -8.819δ

MS – m/z 359.0921 (M^+)

4. 7-chloro-3-(4-(dimethylamino)benzylideneamino)-2-phenylquinazolin-4(3H)-one

IR- C=O – 1661cm^{-1}, C=N – 1591cm^{-1}

^{1}H NMR- Ar (12H) 6.765 – 7.684δ, CH=N -9.659δ, N $(CH_3)_2$ (6H) – 3.031

MS – m/z 402.606 (M^+)

5. 7-chloro-3-(1-(4-hydroxyphenyl)ethylideneamino)-2-phenylquinazolin-4(3H)-one

IR- OH - 2800-3400cm^{-1}, C=O – 1667cm^{-1}, C=N – 1575cm^{-1}

^{1}H NMR- Ar (12H) 7.578 – 8.818δ, OH-10.294 δ, CH_3 (3H) -2.499δ

MS – m/z 389.09 (M^+)

6. 7-chloro-3-(4-hydroxy-3-methoxybenzylideneamino)-2-phenylquinazolin-4(3H)-one

IR- OH - 2800-3400cm^{-1}, C=O – 1668cm^{-1}, C=N – 1588cm^{-1}

^{1}H NMR- Ar (11H) 7.574 – 8.809δ, OH-10.228δ, OCH_3 (3H) – 3.828 δ, CH=N -8.814δ

MS – m/z 405.12 (M^+)

7. 7-chloro-3-(1-(4-chlorophenyl)ethylideneamino)-2-phenylquinazolin-4(3H)-one

IR- C=O – 1679cm^{-1}, C=N – 1583cm^{-1}

^{1}H NMR- Ar (12H) 7.59 – 8.598δ, CH_3 (3H) -2.522δ,

MS – m/z 406.9873 (M^+)

8. 7-chloro-3-(1-(4-nitrobenzylideneamino)-2-phenylquinazolin-4(3H)-one

IR- C=O – 1668cm^{-1}, C=N – 1588cm^{-1}, NO_2 – 1515, 1348cm^{-1}

^{1}H NMR- Ar (12H) 7.592 – 8.062δ, CH=N -8.817δ

MS – m/z 404.1391 (M^+)

9. 7-chloro-3-(1-(4-methoxybenzylideneamino)-2-phenylquinazolin-4(3H)-one

IR- C=O – 1662cm^{-1}, C=N – 1571cm^{-1}

^{1}H NMR- Ar (12H) 7.575 – 8.415δ, CH=N -8.808δ, OCH_3 (3H) – 3.467δ

MS – m/z 389.0875 (M^+)

10.7-chloro-2-phenyl-3-(1-phenylethylideneamino) quinazolin-4(3H)-one

IR- C=O – 1668cm^{-1}, C=N – 1574cm^{-1}

^{1}H NMR- Ar (12H) 7.576 – 8.064δ, CH=N -8.812δ, CH_3 (3H) -2.499δ

MS – m/z 373.33 (M^+)

Anti-TB activity using Alamar Blue Dye [9]

The anti mycobacterial activity of compounds were assessed against M. tuberculosis using microplate Alamar Blue assay (MABA). This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with propotional and BACTEC radiometric method. 200µl of sterile deionzed water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. The 96 wells plate received 100 µl of the Middlebrook 7H9 broth and serial dilution of compounds were made directly on plate.The final drug concentrations tested were 0.01 to 20.0 µl/ml. Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this time, 25µl of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. Blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink.

Results and Discussion

Evaluation of spectral studies

IR spectra: The characteristic absorption peaks were observed for all relevant groups. The absorption peaks around 1600cm^{-1} indicates the formation of C=N ring atoms of Quinazoline, amide N-H stretching vibrations were observed in the region of 3140-3500cm^{-1}. Amide C=O stretching vibrations were observed near 1640-1690cm^{-1} and all other relevant groups absorption were observed for all the synthesized compounds (fig.2). *NMR spectra*: Aromatic protons were observed 6.68- 8.13δ ppm. Amide N-H proton were observed at 6.05-6.40δ ppm, for all the synthesized compounds (fig.3). Molecular ion peak in Mass spectra confirms the molecular weight of the synthesized compounds (fig.4).The C, H and N elemental analysis was calculated by using chem.-ultra software which shows further confirmation of the compounds.

Anti – Tubercular Activity

Anti-TB activity: All the synthesized copounds were evaluated for mycobacterium tuberculosis sudies using microplate Alamar Blue assay (MABA) using Middlebrook 7H9 broth were shown in table-3(fig.4).Among the synthesised compounds CQ-4 was found to be active at 3.125 µl concentration andCQ-1 was found to be active at 6.25 µl concentration&CQ-2,CQ-3 were moderately active at12.5 µl.(fig 4).

Conclusion

A further modification on these molecules can be initiated which would open a new era in developing more therapeutically effective agent against microbial infection of HIV associated tuberculosis in future.

Acknowledgement

The author is grateful to the SIF, IIS Bangalore and SIF, IIT Chennai for NMR, Mass Spectral Studies; Maratha mandals NGH institute Belgaum and the Principal, Management of RVS College of Pharmaceutical Sciences, Coimbatore.

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Table-1 : Substitued Compounds

Comp	R	R_2	R_3
1	H	4-OH	H
2	H	2-OH	H
3	H	H	H
4	H	4-$N(CH_3)_2$	H
5	CH_3	4-OH	H
6	H	3-OCH_3	4-OH
7	CH_3	4-Cl	H
8	H	4-NO_2	H
9	H	4-OCH_3	H
10	CH_3	H	H

Table-2: Physical data of newly synthesized Compounds

Comp	Molecularformula	Molecular weight	Yield%	M.Point(°C)	R_f[a]
1	$C_{21}H_{14}ClN_3O_2$	375.81	89	136	0.61
2	$C_{21}H_{14}ClN_3O_2$	375.81	88	176	0.48
3	$C_{21}H_{14}ClN_3O$	359.81	92	166	0.72
4	$C_{23}H_{19}ClN_4O$	402.88	88	98	0.52
5	$C_{22}H_{16}ClN_3O_2$	389.83	96	160	0.51
6	$C_{22}H_{16}ClN_3O_3$	405.83	84	139	0.41
7	$C_{22}H_{15}Cl_2N_3O$	408.28	82	156	0.42
8	$C_{22}H_{13}ClN_4O_3$	404.81	80	164	0.62
9	$C_{22}H_{16}ClN_3O_2$	389.83	94	185	0.63
10	$C_{22}H_{16}ClN_3O$	373.83	96	170	0.60

Table-3: Anti tubercular activity

Sl. No	Compounds	MIC Activity of antituberculosis (µgm/ml)
1	CQ-5	25
2	CQ-9	25
3	CQ-7	50
4	CQ-2	12.5
5	CQ-3	12.5
6	CQ-8	12.5
7	CQ-6	25
8	CQ-10	25
9	CQ-4	3.125
10	CQ-1	6.25

SCHEME

Synthetic route to obtain target compounds from substituted anthranilic acid

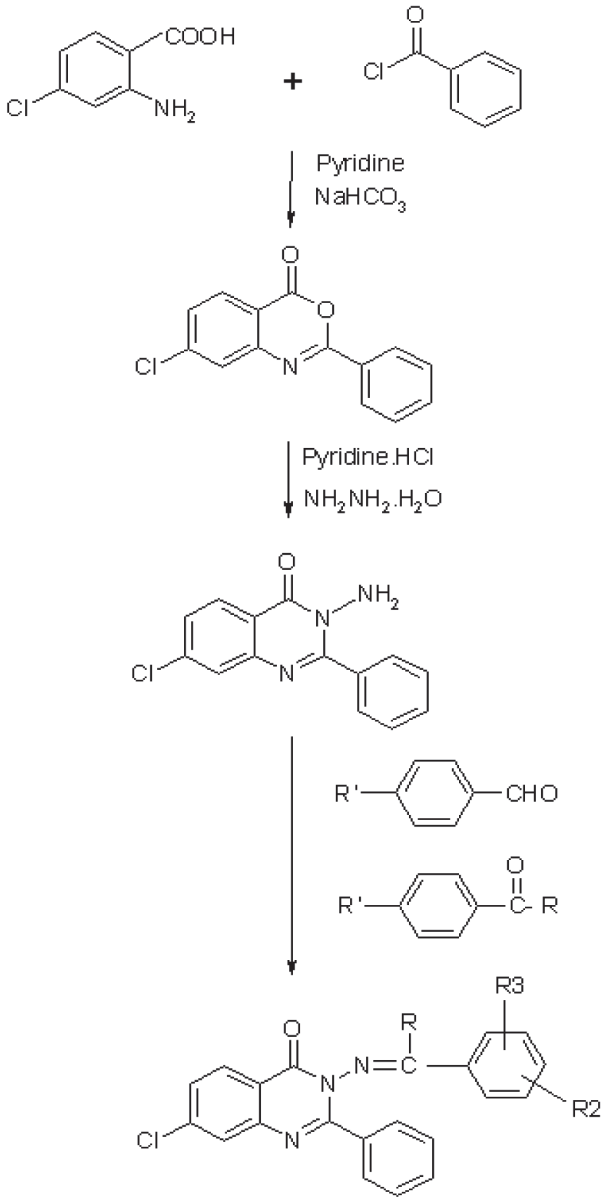
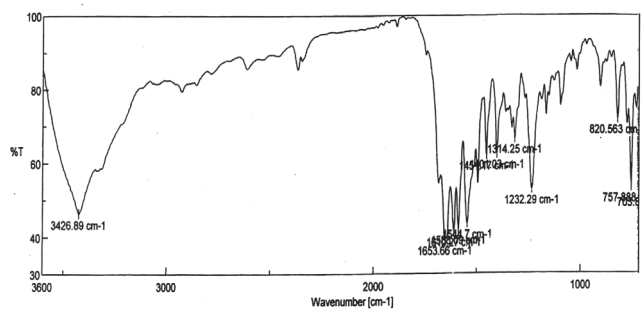
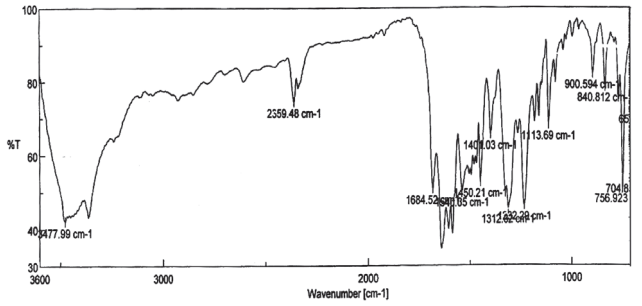


Fig. 1: IR Spectra of Synthesized compounds

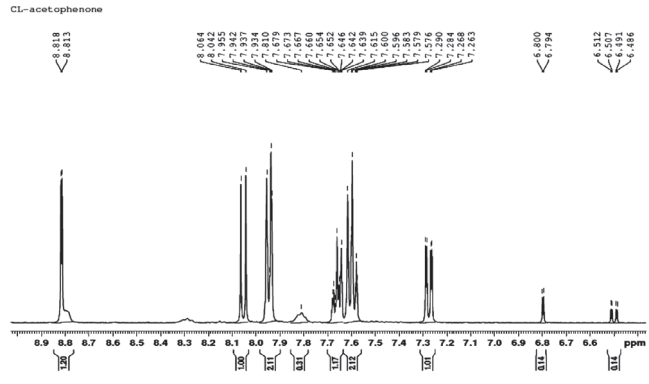


(A) IR Spectra of compound CQ5

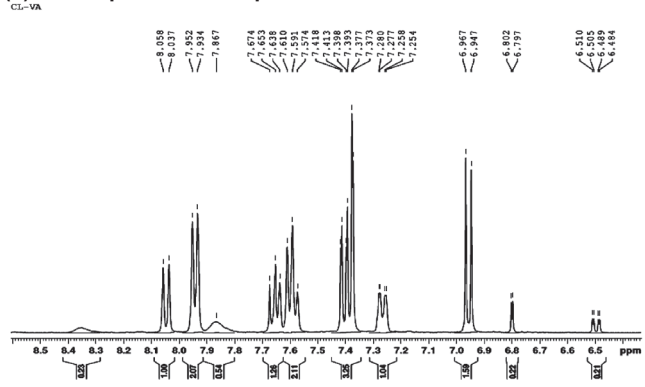


(B) IR Spectra of compound CQ6

Fig. 2: NMR Spectra of synthesized Compounds

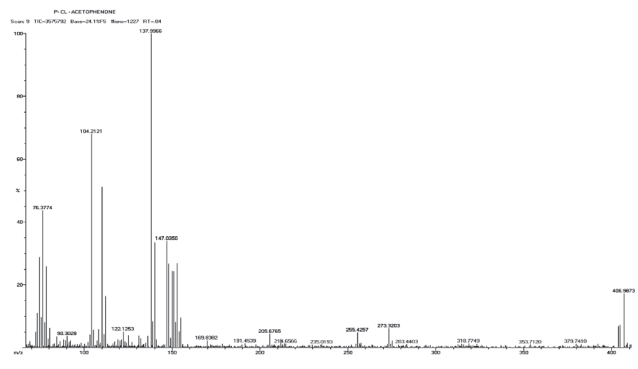


(a) NMR Spectra of compound CQ7

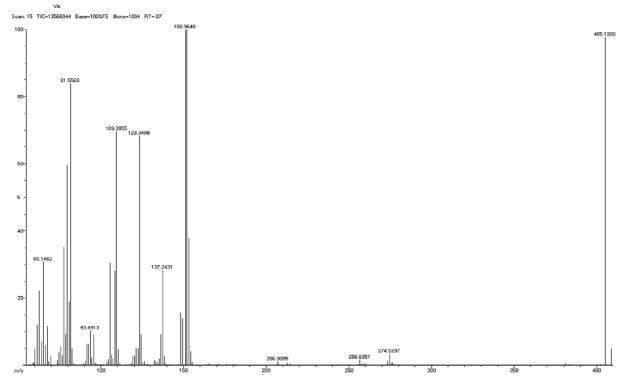


(b) NMR Spectra of compound CQ6

Fig. 3: Mass Spectra of Synthesized Compounds



(a) Mass Spectra of compound CQ7



(b) Mass Spectra of compound CQ6

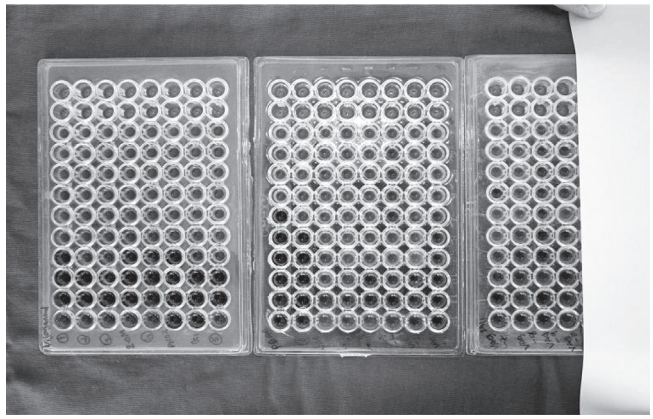


Fig. 4: Anti Tuberculosis Activity of synthesized compounds on microplate Alamar Blue assay (MABA)

Development and Validation of High Performance Liquid Chromatographic Method for Simultaneous Determination of Lamivudine and Zidovudine from Pharmaceutical Preparation

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ABSTRACT

A new, simple, specific, accurate and precise RP-HPLC was developed for simultaneous determination of Lamivudine and Zidovudine in pure and tablet formulations. A thermo C18 BDS column in isocratic mode, with a mobile phase consisting of 0.1 M Ammonium dihydrogen orthophosphate buffer with 1% v/v of tri- ethylamine adjusted to pH 3 by using ortho-phosphoric acid and methanol in the ratio of 60:40 was used for separation. The flow rate was set at 0.6 ml/min and UV detection was carried out at 264 nm. The retention times of Lamivudine and Zidovudine were 4.792 min and 7.750 min respectively. The method was validated for linearity, precision, robustness and recovery. Linearity for Lamivudine and Zidovudine were in the range of 18.75-150 µg/ml and 37.5-300 µg/ml, respectively. Hence it can be applied for routine quality control of Lamivudine and Zidovudine in bulk and pharmaceutical formulations.

Keywords: Lamivudine, Zidovudine, RP-HPLC, Validation

Introduction

Lamivudine, 2′-deoxy–3′-thiacytidine (3TC), a pyrimidine analogue is an oral medication, used for the treatment of infections with the human immunodeficiency (HIV) and Hepatitis B viruses. It works by stopping the spread of the HIV and hepatitis B viruses. Zidovudine (azidothymidine, AZT), 3′-azido-3′deoxythymidine, a synthetic pyrimidine nucleus analogue of the naturally occurring nucleoside thymidine, is an anti-HIV medication, available in capsules, tablets, and syrup formulations and in intravenous form. It prevents HIV by altering the genetic material of healthy T-cells. This prevents the cells from producing new virus and decreases the amount of virus in the body. Both of these anti-retroviral drugs are phosphorylated in the body to their active tri-phosphate form by cellular kinases and selectively inhibit nucleoside reverse transcriptase (RNA dependent DNA polymerase that HIV needs to make more copies of it). Combination of Lamivudine and Zidovudine, as a single tablet at a recommended dose of 150 mg and 300 mg respectively is superior to monotherapy in treatment of HIV infections because, monotherapy with any single antiretroviral agent is no longer considered an acceptable option in the treatment of HIV infection. This combination is also used to treat health-care workers or other individuals exposed to HIV infection after accidental contact with HIV-contaminated blood, tissues or other body fluids [1, 2, 3]. Lamivudine and Zidovudine are officially reported in I.P.[4].

Lamivudine is reported to be estimated by HPLC method for chiral separation [5] .Two similar HPLC methods specify estimation of Zidovudine [6,7]. Only two RP-HPLC method are reported for simultaneous determination in tablet formulation [8,9]. One method is reported for simultaneous assay in combination tablets by derivative spectrophotometry [10].

The aim of the present work is to develop an accurate, new, simple, precise, specific and repeatable method for the determination of 3TC and AZT in pure and its dosage forms. The proposed method was validated as per ICH guidelines [11].

Experimental

High performance Liquid chromatography Shimadzu

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LC 2010 CHT series equipped with quaternary constant flow pump, auto injector with injection volume of 20µl, photo diode array detector, LC 10 software with Thermo BDS C18 column (4.6 mm × 25 cm, 5µm particle size) as stationary phase and a calibrated electronic single pan balance (SARTORIUS AG) were used. Reference standards of Lamivudine and Zidovudine were procured from Cipla Laboratory, Mumbai. Tablets (DUOVIR^{R}) claim for Lamivudine and Zidovudine were 150mg and 300mg/ tablet respectively. All chemicals and reagents used were of AR/HPLC grade.

Preparation of Mobile phase and standard stock solution

The mobile phase was prepared by mixing 600ml of 0.1M Ammonium dihydrogen orthophosphate buffer [$NH_4H_2PO_4$] (pH adjusted to 3 with Ortho-phosphoric acid) with 400ml of methanol. The mobile phase was sonicated 5 minutes and then it was filtered through a 0.45µ membrane filter paper. An accurately weighed quantity of Lamivudine (75mg) and Zidovudine (150mg) were transferred to 100 ml volumetric flask, which was then dissolved and made up to volume with mobile phase. From the above stock solution 0.5, 1, 2, 3, 4ml were diluted to 20ml with mobile phase to give final concentration of 18.75, 37.5, 75, 112.5, 150 µg/ml of Lamivudine and 37.5, 75, 150, 225 and 300 µg/ml of Zidovudine.

Optimised chromatographic conditions

RP- HPLC analysis was performed by isocratic elution with flow rate of 0.6ml/min .The mobile phase containing 0.1 M Ammonium dihydrogen orthophosphate buffer with 1% v/v of tri- ethylamine (pH 3) and methanol in the ratio of (60:40) v/v to obtain well-resolved peaks of Lamivudine (Rt = 4.792 min) and dovudine (Rt = 7.750 min), respectively as shown in Fig. No 1.

Wavelength of maximum absorption was selected by Photo diode Array detector. Both components showed reasonably good response at 264 nm.

Calibration curves for Lamivudine and Zidovudine

Appropriate aliquots from mixed standard stock solution were suitably diluted with the mobile phase in such a way to get concentrations in the range of 18.75 – 300 µg/ml for both the drugs. Twenty µl of working standard solutions were injected in to the column (n=5). Evaluation of two drugs was performed with UV/Vis detector at 264 nm. Peak areas were recorded for all the peaks. The plots of peak area verses the respective concentration of Lamivudine and Zidovudine were found to be linear in the range of 18.75-150 µg/ml and 37.5-300 µg/ ml , respectively. The Calibration curves of Lamivudine and Zidovudine are shown in Fig No:2(a) Fig & No:2(b).

Validation of the method

The developed method was validated in terms of linearity, accuracy, specificity, limit of detection and limit of Quantitation, intra-day and inter-day precision and repeatability of measurement.

Analysis of the marketed formulations

Twenty tablets were weighed accurately and crushed to the fine powder. An accurately weighed quantity of powder equivalent to 150 mg of Lamivudine and 300 mg of Zidovudine transferred to a 100 ml volumetric flask, sonicated for 15 min and made up to volume with mobile phase. Then the solution was filtered through 0.45 ì m filter paper. One ml aliquot from the above solution was transferred into 20 ml volumetric flask and volume adjusted with the mobile phase up to mark to get sample solution. Working sample solutions (n=5) were injected into column at above chromatographic conditions and peak areas were measured. The quantification was carried out by keeping these values to the straight line equation of calibration curve. The results of tablet analysis are shown in Table. No: 3.

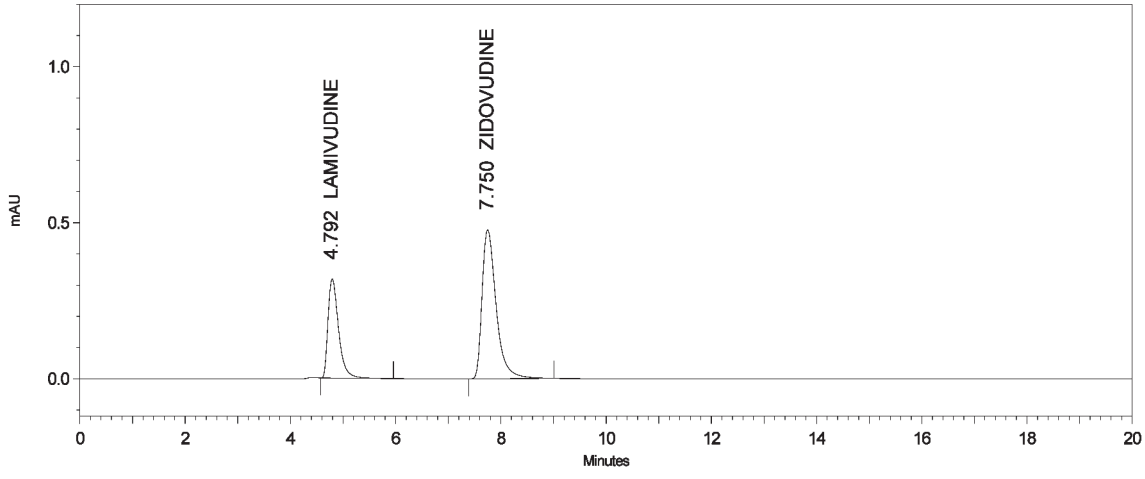

Fig.1: Typical Chromatogram of Lamivudine and Zidovudine by HPLC

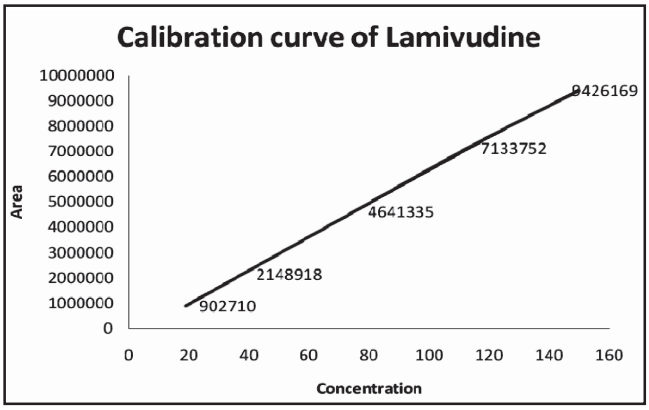

Fig. 2(a) : Calibration curve of Lamivudine

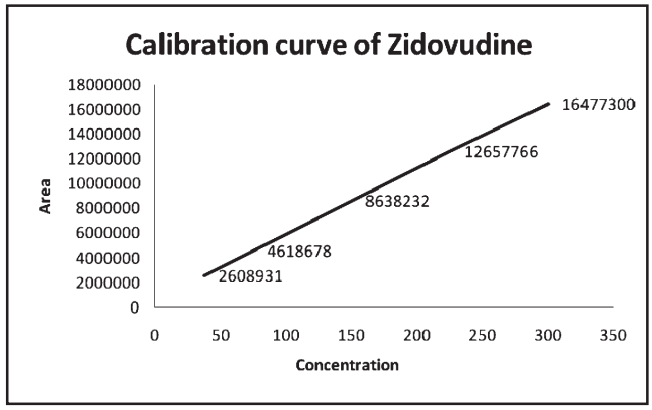

Fig. 2(b) : Calibration curve of Zidovudine

Table-3
Analysis of commercial Tablet (Duovir) (*n=5)

Analyte	Label claim(mg/tablet)	Amount Found(mg/tablet)	C.I.	%RSD
Lamivudine	150	150.230	100.153 ± 0.316	0.254
Zidovudine	300	300.272	100.091 ± 0.1963	0.157

Results and Discussion

Method Development

The method was chosen after several trials with various proportions of buffer (pH 3) and methanol and at different pH values. A mobile phase consisting of buffer (pH 3) and methanol in the ratios of 60:40 was selected to achieve maximum separation and sensitivity. The flow rate 0.6 ml/min and a Thermo BDS C18 column of 4.6 µ particle sizes, a detection wavelength of 264 nm and an injection volume of 20µl and 25^0 C temperatures for the HPLC system were found to be the best for the analysis.

Table 1
System suitability results are as follows

Parameter	Lamivudine	Zidovudine
Retention time (R_t)	4.792	7.75
Asymmetric factor (As)	1.613	1.523
Theoretical plate	2899.9733	4625.3733
Resolution (Rs)	0	7.3
Limit Of Detection (µg/ml)	0.0284	0.0472
Limit Of Quantitation (µg/ml)	0.0876	0.1430

Method validation

The proposed method has been validated for the simultaneous determination of Lamivudine and Zidovudine in bulk as well as tablet dosage form using following parameters.

Specificity

The peak purity of Lamivudine and Zidovudine were assessed by comparing the retention time (R_t) of standard Lamivudine and Zidovudine. Good correlation was also found between the retention time of standard and sample of Lamivudine and Zidovudine.

Linearity

Linearity was studied by preparing different concentration levels. The linearity ranges for Lamivudine and Zidovudine were found to be 18.75-150 µg/ml and 37.5-300 µg/ml respectively. The regression equations for Lamivudine and Zidovudine were found to be y = 65, 228.6784x - 286,181.6220. and y = 52,975.9363x + 656,471.4390. Correlation-coefficient (r2) 0.9997 and 0.9999 respectively.

Table 2
Linearity of Lamivudine and Zidovudine

Sl. NO.	Concentration of Lamivudine (µg/ml)	Peak area	Concentration of Zidovudine (µg/ml)	Peak area
1	18.75	902710	37.5	2608931
2	37.5	2148918	75	4618678
3	75	4641335	150	8638232
4	112.5	7133752	225	12657766
5	150	9426169	300	16477300

Precision

Precision study was performed to find out intra-day and inter-day (within a week) variations in the estimation of Lamivudine and Zidovudine of different concentrations with the proposed method. Percentage relative standard deviation (%RSD) was found to be less than 2% for within a day and day to day variations, which proves that method is precise. Results are shown in Table No: 3.

Accuracy

It was found out by recovery study using standard addition method. Known amounts of standard Lamivudine and Zidovudine were added to pre-analyzed samples at a level from 80 % upto 120% and then subjected to the proposed HPLC method .Results of recovery studies are shown in Table 4.

Stability study

Stability of reagents, mobile phase and sample preparation was studied for 48 hours and compared with freshly prepared solutions , which was found to be stable.

Robustness

It was done by making small changes in the chromatographic conditions and found to be unaffected by small changes like ± 0.1 changes in pH and 2% change in volume of the mobile phase .

Conclusion

The modalities adopted in experimentation were successfully validated as per analytical procedures laid down in routine. The proposed method was validated by preliminary analysis of standard sample and by recovery studies. The percentage of average recoveries was obtained in the range of 99 to 100. The results of analysis of average recoveries obtained in each instance were compared with the theoretical value of 100 percent by means of Student's't' test. As the calculated 't' values are less than theoretical 't' values (Table : 4), it is concluded that the results of recoveries obtained in agreement with 100 percent for each analyte . The absence of additional peaks in the chromatogram indicates non-interference of the common excipients used in the tablets. This demonstrates that the developed HPLC method is simple, linear, accurate,

Table-3

Precision data for the proposed method

Sl. No.	Intra-day measurement				Inter-day measurement			
	3TC		AZT		3TC		AZT	
	Conc(µg/ml)	%RSD	Conc(µg/ml)	%RSD	Conc(µg/ml)	%RSD	Conc(µg/ml)	%RSD
1	37.5	0.53	75	0.392	37.5	0.758	75	0.4
2	75	0.282	150	0.222	75	1.051	150	0.41
3	112.5	0.314	225	0.132	112.5	0.645	225	0.203
4	150	0.176	300	0.298	150	0.201	300	0.328

(n=5)

Table-4

Recovery study of 3TC and AZT

Analyte	Formulation concentration (µg/ml)	Pure drug added (µg/ml)	C.I.	%RSD	%SE	t
3TC	37.5	30	99.813 ±0.482	0.389	0.173	1.077
	37.5	37.5	100.053±1.453	1.169	0.523	0.101
	37.5	45	100.382±1.278	1.025	0.460	0.829
AZT	75	60	100.039±0.224	0.186	0.083	0.492
	75	75	100.029±0.281	0.226	0.101	0.288
	75	90	100.302±0.608	0.487	0.218	1.379

SD: Standard deviation, % SE: Percent standard error, C.I.: Confidence Interval within which true value may be found at 95% confidence level = $R \pm ts\sqrt{n}$, R: Mean percent result of analysis of Recovery study (n = 5). Theoretical 't' values at 95% confidence level for n - 1 degrees of freedom t (0.05, 4) = 2.776.

sensitive and reproducible. Thus, the developed method can be easily used for the routine quality control of bulk and tablet dosage form .

Acknowledgments

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Optimized and Validated Spectrophotometric Methods for The Determination of Aripiprazole Using Ferric Chloride Based on Complexation Reactions

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ABSTRACT

Three simple and sensitive spectrophotometric methods (A-C) were developed for assay of aripiprazole (**APZ**) in pharmaceutical formulations. Methods A and Method B are based on the oxidation of APZ with Fe (III) and the estimation of reduced Fe(III) after chelation with either *o*-phenanthroline (*o*-phen) or bipyridyl (bipy) in the presence of acetate buffer solution. The absorbance's of the colored complexes were measured at 508 nm or 519 nm for Method A and Method B respectively. Method C is based on the oxidation of APZ by Fe (III) in acidic medium, and the subsequent interaction of iron (II) with ferricyanide to form prussian blue, with the product exhibiting an absorption maximum at 796nm. Beer's law is obeyed over the ranges 0.5-7.0 ìg/mL, 0.5-7.0 µg/mL and 0.5-9.0 µg/mL for methods A, B and C, respectively. The calculated molar absorptivity values are 8.88 $x10^4$, 7.21 $x10^4$ and 7.74 $x10^4$ L/mol/cm for method A, B and C, respectively, and the corresponding sandal's sensitivities are $5.0x10^{-3}$, 6.2 $x10^{-3}$and 5.7 $x10^{-3}$ µg/cm^2. The results of the proposed procedures were validated statistically according to ICH guidelines. The proposed methods were applied successfully for the determination of APZ in tablets.

Keywords: Aripiprazole, determination, spectrophotometry, oxidation, tablet forms.

Introduction

Aripiprazole is a quinolinone derivative with the chemical name 7-[4-[4-(2, 3- dichlorophenyl)-1-piperazinyl] butoxy]-3, 4-dihydro-2(1H)-quinolinone (Fig 1). It is a psychotropic agent belonging to the chemical class of benzisoxazole derivatives and is indicated for the treatment of schizophrenia. It is a selective monoaminergic antagonist with high affinity for the serotonin Type 2 (5HT2), dopamine Type 2 (D2), 1 and 2 adrenergic and H1 histaminergic receptors. The different analytical techniques reported so far for the determination of this drug and its metabolites in biological samples as well as in pharmaceutical formulations include LC-MS-MS previously published [1-3]. The determination of aripiprazole in plasma by RP-LC [4] and that in bulk drug, solid dosage forms and related substances by RP-LC [5-8] were also reported. As far as sensitive and economical methods of assay are concerned, very few UV-Visible spectrophotometric methods have been reported for the quantification of aripiprazole based on extractable ion pair complexes [9-10] and UV spectrophotometric method [11]. Literature survey revealed that no attempt was made for the determination of APZ by Fe (III) in the presence of 1, 10-phenanthroline or 2, 2-bipyridyl or potassium ferricyanide. In view of this, we report three simple and sensitive spectrophotometric methods for the assay of APZ in pharmaceutical formulations are reported in this article. The methods are based on the reducing property of the cited drug by iron (III). The formed iron (II) is interacted with 1, 10-phenanthroline, 2, 2'-bipyridyl or ferricyanide to give colored species whose absorbance's were measured at 508, 519 and 796 nm respectively. Such reaction schemes have successfully been used for spectrophotometric determination of many pharmaceutical compounds [12-14]. The developed methods are simple, accurate, precise and sensitive for the determination of APZ in tablets.

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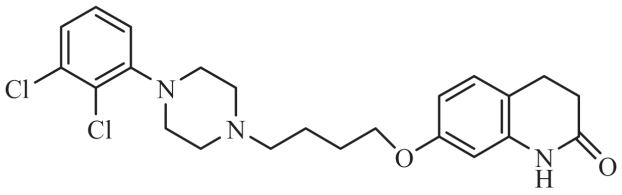


Fig.1: Chemical structure of Aripiprazole

Experimental

Apparatus

All absorption spectra were recorded using UV-Vis-NIR spectrophotometer (Shimadzu 1601, Japan) equipped with 1 cm matched quartz cells by using a personal computer loaded with the UV-PC 3.9 software package. An electronic microbalance (Sartorius MC 5, Germany) was used for weighing the solid materials. A digital pH meter 802 (Systronic, India) was used for measuring the pH of solutions.

Materials and Reagents

All solvents and reagents used were of analytical grade. Double-distilled water was used throughout the investigation. The aqueous solution of 0.05 M ferric chloride (S.D. Fine Chem., Mumbai, India) was prepared by dissolving 0.8110 g of the chemical in 100 ml of distilled water and stored in a dark bottle. The stock solution was then diluted appropriately with distilled water to get 0.005 M working concentration for Method A, Method B and Method C. The solution was prepared afresh just before the experiment.

o-phenanthroline (0.02M) was prepared by dissolving 0.3960 g of *o*-Phenanthroline (S.D. Fine Chem., Mumbai, India) in 100 mL of distilled water.

2, 2' bipyridyl (0.02M) was prepared by dissolving 0.3120 g of 2, 2' bipyridyl (E-Merck., Mumbai, India) in 100 mL of distilled water. Potassium ferricyanide (0.01M) was prepared by dissolving 0.3293 g of potassium ferricyanide (S.D. Fine Chem., Mumbai, India) in 100 mL of distilled water. The stock Potassium ferricyanide solution was then diluted appropriately with distilled water to get 0.002 M working concentration for method C. Sodium acetate- acetic acid buffer of varying pH [3-6.0] values were prepared by mixing appropriate volumes of 0.1M of sodium acetate (S.D. Fine Chem., Mumbai, India) and acetic acid (S.D. Fine Chem., Mumbai, India). The pH was adjusted with the aid of pH-meter.

A gift standard APZ sample was obtained from Inogent laboratories, Hyderabad, India. Three brand tablets were purchased from local medical shop. A stock standard solution containing 500µg $mL^{-1}$of APZ was prepared by dissolving accurately weighed 0.025 g of pure drug in a 50 mL of calibrated flask with methanol. The solution was further diluted with methanol to get working concentrations of 50 µg mL^{-1} APZ for all three methods.

Method A

Different aliquots of APZ solution corresponding to 0.5-7.0 µg mL^{-1} were transferred into a series of 10 mL volumetric flasks. To each flask 1.0ml of acetate buffer (pH 5), 2.0ml of 0.005M ferric chloride and 3.0 mL of o-Phenanthroline (0.02M) were added and kept in a water bath (75 ± 2 ^{0}C) for 20min, then immediately cooled to room temperature (27 ± 1^{0}C) using cold water and the solutions were made up to volume with distilled water. The absorbance of each solution was measured at 508 nm against the reagent blank. The calibration graph was constructed by plotting the absorbance versus concentration of the drug. The concentration of the unknown was read from the calibration graph or computed from the regression equation.

Method B

Varying volumes of APZ solution corresponding to 0.5-7.0 µg mL^{-1} were transferred into a series of 10 mL volumetric flasks. To each flask 1.5 mL of acetate buffer (pH 4), 2.5mL of 0.005M ferric chloride and 3.0 mL of 2,2' bi-pyridyl (0.02M) were added and kept in a water bath (75 ± 2 ^{0}C) for 25min, then immediately cooled to room temperature (27 ± 1^{0}C) using cold water and the solutions were made up to volume with distilled water. The absorbance of each solution was measured at 519 nm against the reagent blank. The calibration graph was constructed by plotting the absorbance versus concentration of the drug. The concentration of the unknown was read from the calibration graph or computed from the regression equation

Method C

Aliquot of a solution containing 0.5-9.0 µg mL^{-1} of APZ were transferred into a series of 10mL standard flasks. To each flask 3.0mL of ferric chloride (0.005M) was added and kept in a water bath (75 ± 2 ^{0}C) for 30min. the flasks were then added each with 3.0 ml of potassium ferricyanide and 1.0 ml of 1N HCl, cooled to room temperature, diluted to mark with distilled water and mixed well. The absorbance of the resulting solution was measured at 796 nm for APZ against a reagent blank prepared similarly. The concentration of the unknown was read from the calibration graph or computed from the regression equation.

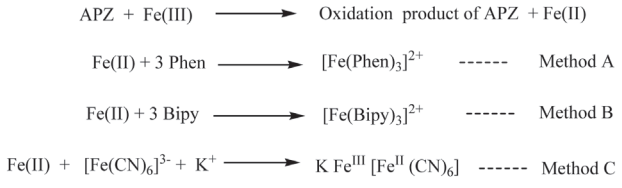
Procedure for pharmaceutical formulations

APZ containing ten tablets were weighed and ground into a fine powder. An amount of the powder equivalent to 25 mg of APZ was weighed into a 50-mL volumetric flask, 30 mL methanol added and shaken thoroughly for about 20 min, diluted to the volume with the same solvent, mixed, and filtered using Whatmann No. 41 filter paper into a 50-mL volumetric flask. The filtrate was further diluted with methanol to get concentration of 50 µg mL^{-1} of APZ for Method A, Method B and Method C. A suitable aliquot was then subjected to analysis.

Results and Discussion

It is known that 1, 10-phenanthroline and 2, 2-bipyridyl form highly stable, intensely red colored, water-soluble chelates with iron (II). The complex formation stabilizes the iron (II) oxidation state, as the formal redox potential of complexes (+1.06 V) is higher than that of the iron aqua-complex. These complexes were applied to the determination of reducing substances by measuring the amount of ferrous iron produced when the analyte solution is treated with an excess of ferric salt. Trace amounts of the reductants are conveniently measured by this way through spectrophotometric determination of ferrous iron using 1, 10- phenanthroline or 2, 2-bipyridyl.

The proposed methods involve the oxidation of APZ with ferric chloride and subsequent complexation of resulting Fe (II) with 1, 10-phenanthroline or 2, 2-bipyridyl to form a red-colored complex, $[Fe\ (phen)_3]^{2+}$, with an absorption maximum at 508 nm or $[Fe\ (bipy)_3]^{2+}$ exhibiting an absorption maximum at 519 nm. The general reaction path of proposed methods is represented in scheme 1. The absorption spectra of colored complexes are shown in Figs 2 and 3.



Scheme-1

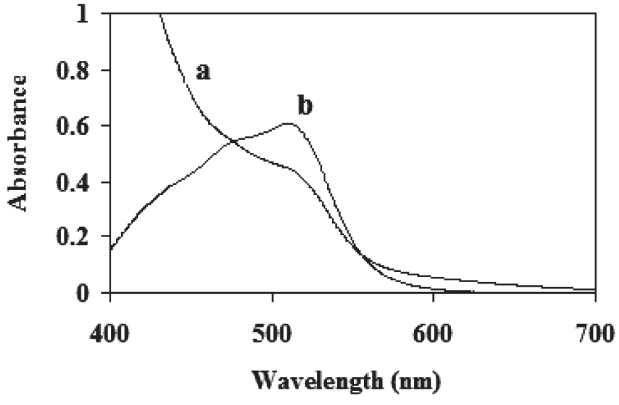


Fig.1: Absorption spectra. (b) Product of APZ (3 µg mL^{-1})-Fe (III)-Phen against reagent blank and (a) reagent blank against water

Optimum reaction conditions

The optimum reaction conditions for the quantitative estimation of APZ were established via a number of preliminary experiments

Effect of pH

The effect of pH for the quantitative determination of

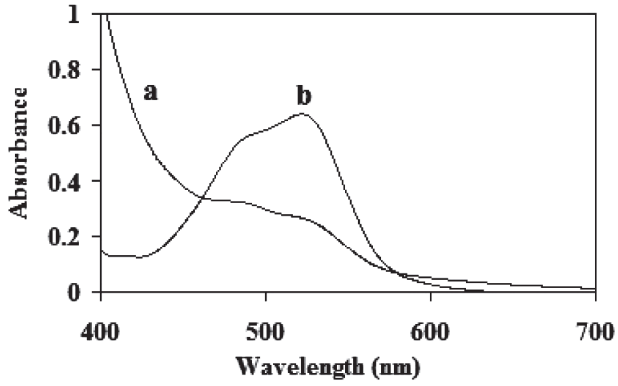


Fig.2: Absorption spectra. (b) Product of APZ (4 µg mL^{-1}) -Fe (III)-Bipy against reagent blank and (a) reagent blank against water

APZ with the proposed oxidation-complexation reaction over the pH range of 3-6 was examined using acetate buffer. At pH<3, the intensity of the absorption of the Fe(II)-phen or Fe(II)-bipy complex decreases most probably due to difficult oxidation of protonated forms of APZ by Fe(III).

An increase in pH >3.0 caused increased absorbance for both methods. So, pH 5.0 and 4.0 were chosen as the optimum pH in Method A and Method B, respectively. The optimum volume of buffer solution required was 1.0 ml in Method A and 1.5 mL in Method B in the total volume of 10 mL.

Effect of reagent concentrations

The effects of reaction variables (methods A and B) such as concentration of Fe (III) and 1, 10-phenanthroline or 2, 22 - bipyridyl have been investigated to develop maximum color by adding to 5.0 µg mL^{-1} of APZ. The effect of iron (III) chloride concentration on color development was studied. These results indicate that maximum absorbance was observed for 2.0 mL of 0.005M of ferric chloride for Method A and 2.5 mL of 0.005M of ferric chloride for method B. Larger volumes of Iron (III) chloride had no effect on the sensitivity of the reaction. Similar observations were made when varying volumes of 0.02M of phenonthroline or bipyridyl solution were added to fixed amounts of APZ (5.0 µg mL^{-1}) and diluted to 10 mL after full color development. It is found that the absorbance reaches its maximum when the amount of reagent is 3.0 mL.

Effects of temperature and heating time

The formation of colored complex was slow at room temperature and required longer time for completion. Hence efforts were made to accelerate by carrying out the reaction at higher temperatures. It was observed that the maximum absorbance was observed after heating the reaction mixture on a water bath at 75°C for about 20 min and 25 min with Fe^{2+}-phen and Fe^{2+}-bipy colored complexes, respectively.

Further heating caused no appreciable change in the color. The color of the complexes formed remained stable at room temperature for more than 12 h.

Method C

Absorption spectra

Fig. 6 shows the absorption spectra of the reaction product of APZ with iron (III) ferricyanide and the reagent blank. The greenish blue product from the studied drug exhibits an absorption maximum at 796 nm. The optimum conditions were established by varying parameters, such as iron (III), ferricyanide, reaction time and acid concentrations.

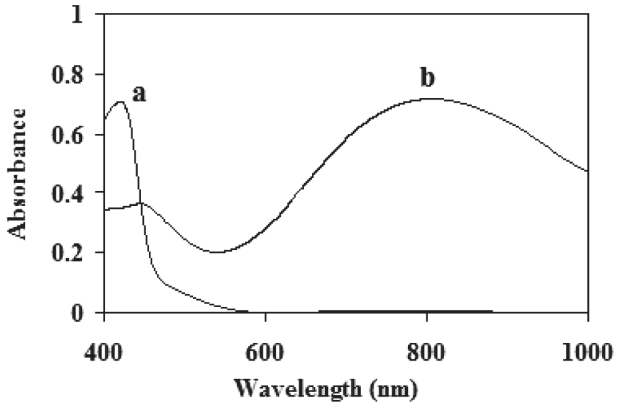


Fig.6: Absorption spectra. (b) Product of APZ (4 µg mL^{-1}) -Fe (III)-K_3 [Fe (CN) $_6$] against reagent blank and (a) reagent blank against water

Optimum iron (III) and ferricyanide concentrations

When a study on the effect of iron (III) chloride concentration on the color development was performed, it was observed that the absorbance increased with increase in the volume of 0.005M iron (III) solution and reaching a maximum upon the addition of 3.0 mL of the 0.005M iron (III) solution to 5.0 µg mL^{-1} of APZ. The influence of the amount of potassium ferricyanide on absorbance was studied. It is found that the absorbance reaches its maximum when the amount of potassium ferricyanide is 3.0 mL.

Effects of temperature and heating time

The color reaction occurred at room temperature, though at high temperature the color developed more rapidly. The absorbance of complex was measured different time intervals at 75± 2^{0}C in water bath. It was observed that the absorbance was maximal when heating time was equal to 30 min. The temperature of 75 ± 2^{0}C and a reaction time of 30 minutes were selected for reproducible results. Further heating caused no appreciable change in the color. The absorbance of the complexes remained constant at room temperature for more than 12 h.

Effect of nature of acid and its concentration

The reaction product, Prussian green, was found to flocculate within 20–30 min of color development. To delay the flocculation, addition of acid after full color development and before diluting to the mark was found necessary. Hydrochloric acid was found to give more stable color and reproducible results compared to sulphuric acid. A 1.0 mL volume of 1N HCl in a total volume of 10 mL was found to be adequate.

Validation of proposed methods

Linearity

Under optimum conditions, a linear relation was obtained between absorbance and concentration of APZ in the range 0.5-7.0 µg/mL in Method A and Method B and 0.5-9.0 µg/mL in Method C. The regression analysis of the plot using the method of least squares was made to evaluate the intercept (a), slope (b), regression coefficient (r^2) and standard deviations of slope and intercept (Table 2). In all cases, Beer's law plots were linear with good correlation coefficients as shown Table 1. The moderately high sensitivity of the method was indicated by the fairly high value of molar absorptivity and low values of sandell sensitivity. The limits of detection (LOD) and limits of quantitation (LOQ) [15] were determined using the formula: LOD or LOQ= k SDa/b, where k=3.3 for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope.

Precision and Accuracy

In order to determine the accuracy and precision of the proposed methods, solutions containing three different concentrations of APZ were prepared within linearity and analyzed in six determinations. The analytical results of precision and accuracy are show in Table .2. The precision of the proposed methods is fairly high, as indicated by the low values of SD and %RSD, respectively.

Accuracy and recovery

The accuracy and reliability of the methods were ascertained through recovery experiments. To a fixed and known amount of drug in the tablet powder, pure APZ was added at two different levels, and the total content was found by the proposed methods. The recoveries of the pure drug added to the tablet powder were shown in (Table.3). The results reveal that the proposed methods are not liable to interference by tablet fillers, excipients and additives usually formulated with pharmaceutical preparations.

Analysis of pharmaceutical formulations

The results (Table 4) indicate that the methods developed compare well with the reference method [11] with respect to accuracy and precision.

Conclusions

The methods proposed are fairly simple and do not require any pretreatment of the drug and tedious extraction procedure. The methods have been demonstrated to be free from rigid experimental conditions. The procedures are

Table-1
Analytical and regression parameters of proposed methods

Parameter	Method A	Method B	Method C
λ_{max} (nm)	508	519	796
Beers law limit (µg/mL)	0.5-7.0	0.5-7.0	0.5-9
Molar absorptivity (l mol^{-1} cm^{-1})	$8.8751x10^4$	$7.2128 x10^4$	$7.7406 x10^4$
Sandell's sensitivity (µg/cm^2)	0.005	0.0062	0.0057
Regression equation (Y = a + bC)			
Slope (b)	0.1979	0.1609	0.1726
Intercept (a)	0.0163	0.0063	0.0133
Correlation coefficient (r^2)	0.9997	0.999	0.9991
Standard deviation of slope (S_b)	$1.4x10^{-3}$	$2.1 x10^{-3}$	$1.79 x10^{-3}$
Standard deviation of intercept (S_a)	$5.87 x10^{-3}$	$8.3 x10^{-3}$	$9.56 x10^{-3}$
Detection limit LOD (µg/mL)	0.098	0.17	0.18
Quantification limit LOQ (µg/mL)	0.297	0.51	0.55

Table 2
Evaluation of precision and accuracy of proposed methods

Proposed method	Concentrations (µg/mL)		RSD	R.E
	Taken	Found*±SD	(%)	(%)
Method A	1	1.01±0.025	2.47	1.0
	3	3.03±0.06	1.98	1.0
	6	6.05±0.078	1.29	0.83
Method B	1	0.99±0.018	1.81	-1.0
	3	3.02±0.048	1.59	0.67
	6	6.03±0.078	1.60	0.5
Method C	1	1.01±0.018	1.78	1.0
	3	3.04±0.062	2.04	1.33
	7	6.98±0.078	1.12	-0.28

*Mean value of six determinations; SD. Standard deviation; RSD. Relative standard deviation ; RE. Relative error

Table 3
Results of recovery experiments by standard addition method

Proposed methods (%)Recovery * ± SD

Proposed methods	Formulation taken (µg/mL)	Pure drug added (µg/mL)	ARPIT-10	ARIP MT-10	ARPIZOL-10
Method A	3	1.5	99.6±1.83	102.9±1.77	99.22±1.36
	3	3	101.67±1.9	102.16±1.05	99.6±0.86
Method B	3	1.5	101.06±0.35	100.91±2.1	98.48±1.84
	3	3	100.67±1.42	99.84±0.77	100.17±0.57
Method C	4	2	100.81±2.6	98.91±1.4	99.2±1.82
	4	4	100.52±1.93	101.07±2.03	99.07±1.57

**Mean value of three determinations*

Table-4
Application of proposed methods to the determination of APZ in pharmaceutical formulations

Pharmaceutical preparations	# Reference method (%Found* ± SD)	Proposed methods (% Found* ± SD)		
		Method A	Method B	Method C
ARPIT-10[a]	101.81±1.31	100.08±1.94 t=1.81 F=2.14	101.33±1.46 t=0.599 F=1.24	100.12±1.68 t=1.93 F=1.64
ARIP MT-10[b]	101.61±1.79	101.09±1.87 t=0.49 F=1.1	99.95±0.94 t=1.41 F=3.62	101.38±1.95 t=0.12 F=1.18
ARPIZOL-10[c]	99.73±1.18	98.79±1.75 t=0.732 F=2.2	100.3±0.65 t=1.09 F=3.29	100.14±1.5 t=0.52 F=1.61

Marketed by: a. Crescent Pharma, India; b. Torrent Pharma, India; c. Sun Pharma, India. All aripiprazole tablets containing 10 mg per tablet

*Mean value of six determinations. The theoretical values of t (2.57) and F (5.05) at confidence limit at 95% confidence level and five degrees of freedom (p=0.05)

based on well established and characterized redox and complex formation reactions and use cheaper and readily available chemicals. The methods have wider linear range with good accuracy, precision and stability of the colored species for ≥ 12 h. Thus, the developed methods are sensitive, selective, offer advantages of reagent availability, stability, less time consumption and free from interferences by common additives and excipients qualifying them for the analysis of aripiprazole in pharmaceutical formulations.

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Studies on Antimicrobial Screening of Some Quinoxaline Derivatives and their Metal Complexes

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ABSTRACT

A few quinoxaline derivatives and their VO(IV), Cr(III), Mn(II), Fe(III), Co(II), Ni(II), Cu(II), Zn(II), Cd(II) and Hg(II) complexes have been screened for antimicrobial activity against some gram (+ve) and gram (-ve) bacterial and fungal species. The ligands and their metal complexes exert differential activity against the microorganisms studied and the results obtained are discussed.

Key words: *Metal–Quinoxaline derivatives, antimicrobial activity.*

Introduction

In the domain of organic compounds of biological significance, quinoxalines and their derivatives command for themselves an appropriate position, for these compounds are endowed with antimicrobial, antitubercular, antihistamic, anticancer, cytotoxic, etc. activities[1-4]. The preparation and study of metal complexes with such biologically relevant compounds is prompted by the important role played by metal ions in a variety of biochemical processes. Such model studies attempt to provide low molecular weight species which mimic both the structure and reactivity of metal ion sites in complex biological systems. The biological and medicinal potency of metal complexes has been established by various types of activity [5, 6].

Considering these aspects, the biological activity studies have been undertaken in which some quinoxaline derivatives and their metal complexes have been screened for their antimicrobial activity. In this paper, we report the results on the antimicrobial screening of quinoxaline derivatives namely 2–hydroxybenzaldehyde-2-(3-chloro-2-quinoxalinyl)semicarbazone(HBCQS),2-hydroxy-3-ethoxybenzaldehyde-2-(3-chloro- 2-quinoxalinyl) semicarbazone (HMCQS) and 2-furaldehyde-2-(3-chloro-2-quinoxalinyl)hydrazone (FCCQH) and their VO(IV), Cr(III), Mn(II), Fe(III), Co(II), Ni(II), Cu(II), Zn(II), Cd(II) and Hg(II) complexes against the bacteria : Bacillus subtilis (Gram +ve) and Escherichia coli (Gram -ve) and against the fungus: Fusarium oxysporum.

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Experimental

Preparation of the quinoxaline derivatives and their metal complexes

All the chemicals used were of AR or BDH grade. The precursors: 2, 3-dichloroquinoxaline, aldehyde semicarbazones and 3-chloro-2- hydrazinequinoxaline were prepared as reported earlier [7-10]. HBCQS and HMCQS were synthesized by refluxing equimolar methanolic solutions of 2,3-dichloroquinoxaline and the respective aldehyde semicarbazone for 2 hrs and the synthesis of FCCQH was accomplished by stirring a solution of 2-furaldehyde in DMF with 3-chloro-2-hydrazinequinoxaline in 1:1 mole ratio for 2hrs. The yellow solids separated out were recrystallized from methanol/ $CHCl_3$-hexane; m.p. HBCQS 212, HMCQS 215 and FCCQH 129^{0}C. In the preparation of metal complexes, the metal (in the form of a suitable salt) and the quinoxaline derivative were combined in 1:2 mole ratio using methanol or aqueous methanol for the metal salts and methanol for the derivatives. The contents were refluxed on a water bath for about 3hrs, the solid that separated was washed with water, hot methanol and ether and dried in air.

Preparation of test samples

The antibiotics streptomycin sulphate was used as standard for antibacterial screening and Kanamycin for antifungal screening. All the test samples and the standards under present investigation were dissolved in DMF to give required concentrations.

Preparation of inoculum and nutrient medium

Nutrient broth (pH-7.2) was used for the preparation of inoculum of bacteria. The composition of broth was peptone 5.0 g, beef extract 1.5g, yeast extract 1.5g and distilled water 1000 ml. Nutrient broth was used for the preparation of medium for antibacterial screening. The medium contained 1.5% of agar in addition to the composition of nutrient broth [11]. For antifungal screening, inoculum was prepared by transferring a loopful of stock culture (Glucose 500mg, KNO_3 0.175mg, KH_2PO_4 0.075mg, $MgSO_4.7H_2O$ 0.0075mg, Agar-Agar 1.5g, distilled water 1000ml) to a 125 ml Erlenmayer flask containing 80ml of Asthana and Hawker's broth. The composition of inoculum broth is same as that of stock culture with the exception of agar. The inoculum flask was incubated for 18 hours at 25°c and stored at 5°c.

Preparation of plates

For antibacterial screening, the agar medium was sterilized by autoclaving at 121°c (15lb/sq. In.] for 15 minutes. The petri dishes and pipettes plugged with cotton were sterilized in an oven at 150°c for one hour. About 25ml of the molten agar medium was poured in each of the sterilized petri dishes. For bioassays, a suspension of approximately $1.5x10^8$ bacterial cells/ml in sterile normal saline was prepared as described by Forbes et al[12]. About 1.5ml of it was uniformly spread on nutrient agar medium in 12x1.2 cm sterilized glass petri dishes, kept aside for 15 min, and excess of suspension was then drained. The medium containing inoculum was allowed to solidify at room temperature. After solidification of the medium, cups each of diameter 8 mm were made about 2 cm apart using sterile cork borer at equal distances. For antifungal activity, the sterile petri plates were used for investigation. About 20 ml of priorly inoculated Asthana & Hawker's agar medium was poured in them. The medium containing inoculum was allowed to solidify at room temperature. After solidification of the medium, cups (diameter 8 mm) were made with the help of a sterile borer at equal distances.

Measurement of activity

Three different concentrations of test samples and standard antibiotics 200, 400 and 600µg/ml in the case of antibacterial assay and 200, 600 and 800µg/ml in the case of antifungal assay were employed. Accurately measured test and standard solutions were placed in cups prepared in seeded agar petri plates. The petri plates were left undisturbed in a cool place for one hour to allow proper diffusion and then incubated at 37^0C for 48 hrs in the case of bacteria and at 25^0C for 5 to 7 days in the case of fungus. After the incubation period, the diameter of zone of inhibition was measured. The experiments were carried out in duplicate.

Results

The results of the antibacterial and antifungal screening of the compounds are presented in Tables 1 and 2 respectively. A value of zone of inhibition (in mm) <5 has been considered marginal, 5-12 moderate and >12 significant. It may be seen from the tables that the activity profiles of the quinoxaline derivatives and their metal complexes are varying. Further, it may be noted that while the semicarbazone derivatives and their metal complexes perform better than their hydrazone counterparts with respect to antibacterial activity, opposite is the case with antifungal activity. Antibacterial activity, results on the antibacterial activity of the compounds screened indicate that HBCQS is active against both the bacteria whereas HMCQS exerts its activity only against Escherichia coli and FCCQS against neither. As for the metal complexes, while Hg-HBCQS and Ni-, Cu- and Cd-HMCQS complexes show significant activity against both the bacteria, Mn- and Fe-HMCQS and Hg-FCCQS do so only against Bacillus subtilis and Fe- and Ni-HBCQS and VO- and Zn-HMCQS only against Escherichia coli. The activity of the complexes increases, though irregularly, with increase in concentration. Antifungal activity, with regard to antifungal activity, the ligands and most of the complexes are active only at highest concentration level. On the contrary, the Co, Ni, Zn, Cd and Hg complexes of FCCQH exhibit significant activity at all the concentration levels studied.

Discussion

The activity of the ligands may be attributed to their chelating with the metal ions in the biosystem. It may be inferred from the results that the metal complexes fare better than the ligands. There are reports that antimicrobial activity of organic compounds is considerably enhanced by complexation with metal ions [13, 14]. A possible mode of toxicity can be forwarded in the light of chelation theory [15]. Chelation lowers the polarity of the metal ion considerably, due, mainly to the partial sharing of its positive charge with donor groups and possible π-electron delocalization over the whole chelate ring. The reduced polarity of the metal ion, in turn, increases its lipophilic character. This favours interaction with moieties of cell wall and membrane. This increased interaction, most probably, leads to the breakdown of permeability barrier of the cell, resulting in interference with the normal cellular processes i.e. toxicity.

However, chelation is not the only criterion for antimicrobial activity. Factors such as nature of the metal ion, geometry of the complex, steric and pharmacokinetic aspects, etc. also play an important role in deciding the antimicrobial potency of a compound[16]. The inactivity or marginal/significant activity of the present compounds may be attributed to these reasons. For example, if the geometry and charge distribution of a compound is incompatible with that of the microbial cell walls, then compound can not penetrate the wall and hence the toxicity is not manifested. However, accurate information regarding the mode of action of a compound can be arrived at only after identifying the active species of the compound and the site of its action.

Table-1
Antibacterial activity of the compounds

Sl.No.	Compound	Zone of inhibition in mm					
		Bacillus subtilis			*Escherichia coli*		
		A*	B*	C*	A*	B*	C*
1	HBCQS	13	15	17	12	19	24
2	HMCQS	—	—	—	16	26	28
3	FCCQH	—	—	—	—	—	—
4	VO-HBCQS	2	4	7	—	—	3
5	Mn- HBCQS	—	—	—	10	13	14
6	Fe- HBCQS	8	10	12	13	14	16
7	Co- HBCQS	8	12	17	3	4	4
8	Ni- HBCQS	—	—	—	15	16	17
9	Cu- HBCQS	2	4	9	—	3	4
10	Zn- HBCQS	7	12	14	1	3	7
11	Cd- HBCQS	2	4	9	1	3	8
12	Hg- HBCQS	13	14	17	20	21	24
13	VO-HMCQS	—	—	—	12	18	26
14	Mn- HMCQS	18	20	23	10	13	15
15	Fe- HMCQS	16	19	21	11	14	18
16	Co- HMCQS	—	—	—	10	15	18
17	Ni- HMCQS	14	18	20	15	18	21
18	Cu- HMCQS	16	19	24	15	19	23
19	Zn- HMCQS	—	—	—	18	27	29
20	Cd- HMCQS	22	23	28	16	20	26
21	Hg- HMCQS	—	—	—	—	—	—
22	VO-FCCQH	—	—	—	6	8	9
23	Cr-FCCQH	4	5	5	3	3	4
24	Mn-FCCQH	—	—	—	—	—	—
25	Fe-FCCQH	—	—	—	—	—	—
26	Co-FCCQH	—	—	—	—	—	—
27	Ni-FCCQH	3	5	6	—	—	—
28	Cu-FCCQH	4	6	7	2	2	2
29	Zn-FCCQH	3	6	8	6	9	11
30	Cd-FCCQH	3	4	4	—	—	—
31	Hg-FCCQH	19	21	25	9	14	16
32	Streptomycin Sulphate	14	17	19	14	18	21

Test solution and standard solution A*-200 µg/ml, B*-400 µg/ml, C*- 600 µg/ml.

Table-2

Antifungal activity of the compounds

Sl. No.	Compound	Zone of inhibition in mm *Fusarium oxysporum*		
		A*	B*	C*
1	HBCQS	—	6	10
2	HMCQS	—	—	10
3	FCCQH	—	6	7
4	VO-HBCQS	—	—	6
5	Mn- HBCQS	6	13	17
6	Fe- HBCQS	—	6	11
7	Co- HBCQS	—	—	—
8	Ni- HBCQS	—	6	15
9	Cu- HBCQS	6	13	15
10	Zn- HBCQS	—	—	6
11	Cd- HBCQS	—	6	14
12	Hg- HBCQS	6	11	15
13	VO-HMCQS	—	—	6
14	Mn- HMCQS	—	11	14
15	Fe- HMCQS	—	11	15
16	Co- HMCQS	—	—	—
17	Ni- HMCQS	—	6	13
18	Cu- HMCQS	—	6	12
19	Zn- HMCQS	—	—	6
20	Cd- HMCQS	—	6	12
21	Hg- HMCQS	6	13	16
22	VO-FCCQH	8	10	14
23	Cr-FCCQH	3	5	6
24	Mn-FCCQH	3	5	5
25	Fe-FCCQH	9	13	16
26	Co-FCCQH	14	18	21
27	Ni-FCCQH	14	19	23
28	Cu-FCCQH	8	11	15
29	Zn-FCCQH	18	21	25
30	Cd-FCCQH	19	21	24
31	Hg-FCCQH	20	22	25
32	Kanamycin	8	11	14

Test solution and standard solution A*-200 g/ml, B*-600 µg/ml, C*-800 µg/ml.

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Synthesis and Antimicrobial Screening of Some Semi-synthetic Derivatives of Citral and Camphor

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Abstract

In the present study, synthesis of some novel derivatives of citral and camphor was carried out. The structures of the synthesized compounds were confirmed by IR, MASS and NMR datas. Compounds synthesized were subjected for antibacterial and antifungal studies. The antibacterial activity of synthesized compounds was performed against two gram positive bacteria viz., B.subtilis and S.aureus and two gram negative bacteria viz., E.coli and P. vulgaris by using cup plate method. Ampicillin sodium was employed as standard to compare the results. The fungi employed for screening were A.niger, A.flavus, F.oxysporum and C.verticulata. clotrimazole was used as standard. The result suggests that all the compounds posses' good antibacterial activity but has shown very mild antifungal activity in comparison with the standard drug(s). Among them compound II showed good potency against both gram positive and negative bacteria.

Keywords: citral, camphor, antibacterial, antifungal.

Introduction

In recent years, new plant drugs are finding their way into medicine as purified phytochemicals, rather than in the form of traditional galenical preparations.[1] Aspirin a semisynthetic derivative of salicin is still used as one of the best drug in the world market for its analgesic and antipyretic activity. Aspirin is still used as a best drug for CVS diseases. Many semisynthetic derivatives like quinine, codeine, artemisinin etc. are very potent drugs and still these drugs are in the world market. Thus, An analysis of the origin of the drugs developed between 1981 and 2002 showed that natural products or natural product- derived drugs comprised 28% of all new chemical entities (NCEs) launched onto the market.[2] In addition, 24% of these NCEs were synthetic or natural mimic compounds, based on the study of pharmacophores related to natural products.[3] This combined percentage (52% of all NCEs) suggests that natural products are important sources for new drugs and are also good lead compounds suitable for further modification during drug development. The large proportion of natural products in drug discovery has stemmed from the diverse structures and the intricate carbon skeletons of natural products. Since secondary metabolites from natural sources have been elaborated within living systems, they are often perceived as showing more "drug-likeness and biological friendliness than totally synthetic molecules," [4] making them good candidates for further drug development.[5,6]

Citral also called 3,7-dimethyl-2,6-octadienal, a pale yellow liquid, with a strong lemon odour, that occurs in the essential oils of plants. Citral is also used as a flavor and for fortifying lemon oil. It also has strong anti- microbial qualities[7] and phenomenal effects in insects. Camphor ($C_{10}H_{16}O$) found in wood of the camphor laurel (*Cinnamomum camphora*) is used as antibacterial agent, preservatives, irritant,etc. [8] Since both these drugs were used in our routine life, the main disadvantage was both are volatile in nature.

In the present study, some novel semi-synthetic derivatives of citral and camphor was synthesized and screened for their possible antimicrobial activity.

Materials and Methods

Citral (Gift sample from Director JNTUA-OTRI), Camphor (Finar chemical limited Ahmedabad), Semicarbazine (Sisco research laboratoratories, Mumbai), sodium acetate (S.D-Fine Chem. Ltd, Mumbai), Benzophenone (S.D-Fine Chem. Ltd, Mumbai), Pyridine (Merck specialties pvt.ltd), Ethanol (Changshu yang Yuan

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chemical ltd China), formaldehyde (Merck specialties pvt.ltd), Conc.HCl (Finar chemical limited Ahmedabad), Acetone (S.D-Fine Chem. Ltd, Mumbai), Magnesium (Merck LTD, Mumbai), Diethyl Ether (Finar chemical limited Ahmedabad), Orthophenylenediamine (Oxford lab, Mumbai).

Procedure

The compounds synthesized using the below mentioned procedure was subjected for spectral analysis like FT-IR, MASS and proton NMR. The melting point was determined using open tube capillary method and are uncorrected. The purity of the compounds was determined using Thin Layer Chromatography.

Synthesis of (2-[(2z)-3,7-Dimethylocta-2,6-Dien-1-Ylidene]HydrazineCarboxamide) Compound 1 from Citral[9].

1gm of Semicarbazine and 1.5gm of crystallized sodium acetate was dissolved in 8-10ml of water, to this 0.5gm of the citral was added and shaken for a while. To the above solution alcohol was added until the clear solution was obtained. The mixture was shaken for few minutes and allowed to stand for some time. Semicarbazone crystallized from the cold solution on standing. Filtered off the crystals and washed with cold water and recrystalized from dilute ethanol.

*Spectral datas:*FT-IR N-H Stretching at 3438.41cm^{-1}methyl at 3321.52 cm^{-1}, C-H stretching salicylic at 2922.53 cm^{-1}, conjugate C=C at 1644.cm^{-1},NO_2 at 1583.12 and 1329.20 cm^{-1}. ^{1}HNMR (δ ppm) at 7.5 s (1H –CH), 7.0 s (1H-NH), 6.0 s (2H -NH_2), 5.20 t (1H-CH), 2.0 d (4H -CH_2), 1.71 t (9H-CH_3). The mass spectrum of the compound showed its molecular ion peak (M^+) at m/z at 209. It exhibited the fragmentation pattern characteristic of the compound.

Synthesis Of Compound II ((2z)-N-Diphenylmethylidene-3,7-Dimethylocta-2,6-Dienamide) from Citral[10].

A mixture of 0.5gm of citral, 0.5gm of benzophenone oxime, 5ml of ethanol and 0.5ml of pyridine was refluxed on a water bath for 30minutes. Ethanol was removed by evaporation in a stream of air. 5ml of water was added to the cooled residue and kept in an ice bath and stirred until the oxime crystallized out. The solid was filtered and washed with a little water and dried. The dried product was recrystalized using ethanol.

Spectral datas: FT-IR peak at 3248(C=C stretching in aromatic compound), 3055(C=C stretching in aromatic compound), 1631(C=O stretching in amide), 1076(C-N stretching), 997.01, 918,766(C-H bending in alkenes), 488(C-C bending).^{1}HNMR (δ ppm) at 7.8 – 7.39m (10 H – Ar-H) 5.81s (1H - =CH), 5.20s (1H- =CH), 2.00t (4H-CH_2), 1.71s (9H – CH_3).The mass spectrum of the compound showed its molecular ion (M^+) peak at m/z at 331. It exhibited the fragmentation pattern characteristic of the compound.

Synthesis Of Compound III N,N'-Bis-[(2E)-1-Methylbicyclo [2.2.1] Hept-2-Ylidene]Benzene-1,2-Diamine-Ethane(1:1) From Camphor[11]

5.3gm of dry aniline, 2gm of powdered par formaldehyde and 6gm of camphor was taken in a 50ml round bottom flask attached to a reflux condenser. 8ml of 95% ethanol to which 2-3 drops of conc.HCl acid have been added was introduced in to the reaction mixture and refluxed on a water bath for 1 hour. The reaction mixture was almost clear and homogenous. The yellowish solution was filtered through a pre heated Buchner funnel and the filterate was transfered to a 100ml wide mouthed conical flask and still warm. 40ml of acetone was added and allowed to cool room temperature and placed on a icebath to obtain crystals. Crystals are filtered at the pump and washed with 2-3ml of acetone. Acetone was drained and crystals are dried in steam oven for 30min.The yield of crude b-dimethylamino propinophenone Hcl, m.p-153-155^{0}c, is 7.4gm. If desired crude product may be recrystallised by dissolving in 9ml of hot rectified spirit and slowly adding 45ml of acetone. Purified material melts at 155-156^{0}c and recovery about 90%.

Spectral datas: FT-IR peak at N-H stretching at 3216.46 and 3080.03 cm^{-1}, C-H stretching at 2913.90 cm^{-1}, C=C ring aromatic at 1437.43 cm^{-1}, C=O 1643.65 cm^{-1}. ^{1}HNMR (δ ppm) at 7.3q (1H-Ar-CH), 1.60-1.34m (8H-CH_2), 1.5t (2H-CH), 1.4-1.2m (4H-CH_2), 1.11m (18H-CH_3).The mass spectrum of the compound showed its molecular ion (M^+) peak at m/z at 376. It exhibited the fragmentation pattern characteristic of the compound.

Synthesis of Compound IV 2-Benzyl-1-Methylbicyclo [2.2.1] Hept-2-Benzoate From Camphor [9]

A solution of ethyl magnesium bromide in 50ml of anhydrous ether was prepared from 27.3gm of ethyl bromide and 6gm of magnesium. After cooling, 25.5 gms phenyl acetylene in 30ml of anhydrous ether was added drop wise. The reaction mixture was gently refluxed for 2hrs and cooled to room temperature. Then the mixture was slowly stirred and a solution of 45gm of benzophenone in 50ml of anhydrous ether was added and continued stirring at room temp for 1.5 hrs. Then the mixture was refluxed for 1hr and cooled in a n ice bath. 55gm of ammonium chloride as a saturated agues solution was added and the product starts liberating out, the residue oil was kept in ice and triturate with light petroleum until the butry phenyl propynol crystallizes out. Recrystallize using a mixture of benzyl and light petroleum. The yield is 35gm, m.p - 78-80.

Spectral datas: FT-IR peak at N-H stretching at 3455.12cm^{-1}, Aromatic C-H bending at 3085.13cm^{-1}, C=0 bending at 1733.75cm^{-1},C-H methyl group at 3324.36, Aromatic C-H bending at 826.26cm^{-1}, aromatic rings at 1497.27^{-1} cm^{-1}. ^{1}HNMR (δ ppm) at 7.99d (2H-Ar-CH), 7.46d (1H-Ar-CH), 7.37d (2H-Ar-CH), 7.21d (2H-Ar-CH), 7.12d (2H-Ar-CH), 7.08d (1H-Ar-CH), 3.06m (2H-CH_2), 1.90q (2H-CH_2), 1.52q (2H-CH_2), 1.49q (2H-CH_2), 1.42d (1H-CH),

1.16s (3H-CH_3), 1.11t (6H-CH_3).The mass spectrum of the compound showed its molecular ion (M^+) peak at m/z at 348. It exhibited the fragmentation pattern characteristic of the compound.

Synthesis Of Compound V N-[(2E)-1-Methylbicyclo [2.2.1]Hept-2-Ylidene]Aniline From Camphor[11]

1 gm of Orthophenyl diamine dissolved in 10ml of ethanol and 1gm of camphor was taken in a 50ml round bottom flask. The mixture was heated for 3hrs on a water bath with occasional stirring. The product precipitates out as fine crystals. The crystals were filtered out and recrystallized using ethanol and dried in vacuum over calcium chloride.

Spectral datas: FT-IR peak of N-H bending at 3441.99cm^{-1}, Aromatic C-H bending at 3061.84cm^{-1}, C=0 stretching at 1733.75cm^{-1}, Aromatic C-H bending at 810.69cm^{-1},aromatic rings at 1497.12.cm^{-1}. ^{1}HNMR (δ ppm) at 7.3t (5H—Ar-CH), 1.6d (4H-CH_2), 1.5m (1H-CH), 1.4d (2H-CH_2), 1,1s (6H-CH_3). The mass spectrum of the compound showed its molecular ion (M^+) peak at m/z at 227. It exhibited the fragmentation pattern characteristic of the compound.

The schematic representation of the above mentioned procedures is illustrated in fig. no. 1.

The compounds synthesized were subjected for physical and spectral analysis.

Antibacterial activity [12]

The antibacterial activity of synthesized compounds was performed against two gram positive bacteria viz., B.subtilis and S.aureus and two gram negative bacteria viz., E.coli and P. vulgaris by using cup plate method. Ampicillin sodium was employed as standard to compare the results.

Solution of the test compounds were prepared by dissolving 10mg each in dimethylformamide (10 ml, AR grade). A reference standard for both gram positive and gram negative bacteria was made by dissolving accurately weighed quantity of ampicillin sodium in sterile distilled water, separately.

The nutrient agar medium was sterilized by autoclaving at 121°C (15 lb/sq. inches) for 15 min. The petriplates, tube and flasks plugged with cotton were sterilized in hot-air oven at 160°C, for an hour. Into each sterilized petriplate (10 cm diameter), about 27 ml of molten nutrient agar medium was poured and inoculated with the respective strain of bacteria (6 ml of inoculum to 300 ml of nutrient agar medium) was transferred aseptically. The plates were left at room temperature to allow the solidification. In each plate, three cups of 6 mm diameter were made with sterile borer. Then 0.1 ml of the test solution was added to the respective cups aseptically and labeled, accordingly. The plates were kept undisturbed for atleast 2 hours in refrigerator to allow diffusion of the solution properly into nutrient agar medium. After incubation of the plates at 37° ± 1°C for 24 hours, the diameter of zone of inhibition surrounding each of the cups was measured with the help of an antibiotic zone reader. All the experiments were carried out in triplicate. Simultaneously, controls were maintained employing 0.1 ml of dimethyl formamide to observe the solvent effects. The results are presented in Tables No. 1.

Antifungal Activity [13]

The fungi employed for screening were A.niger, A.flavus, F.oxysporum and C.verticulata. The solutions of test compounds were prepared by a similar procedure described under the antibacterial activity. Reference standard (1mg/ml conc.) was prepared by dissolving 10 mg of

Table 1

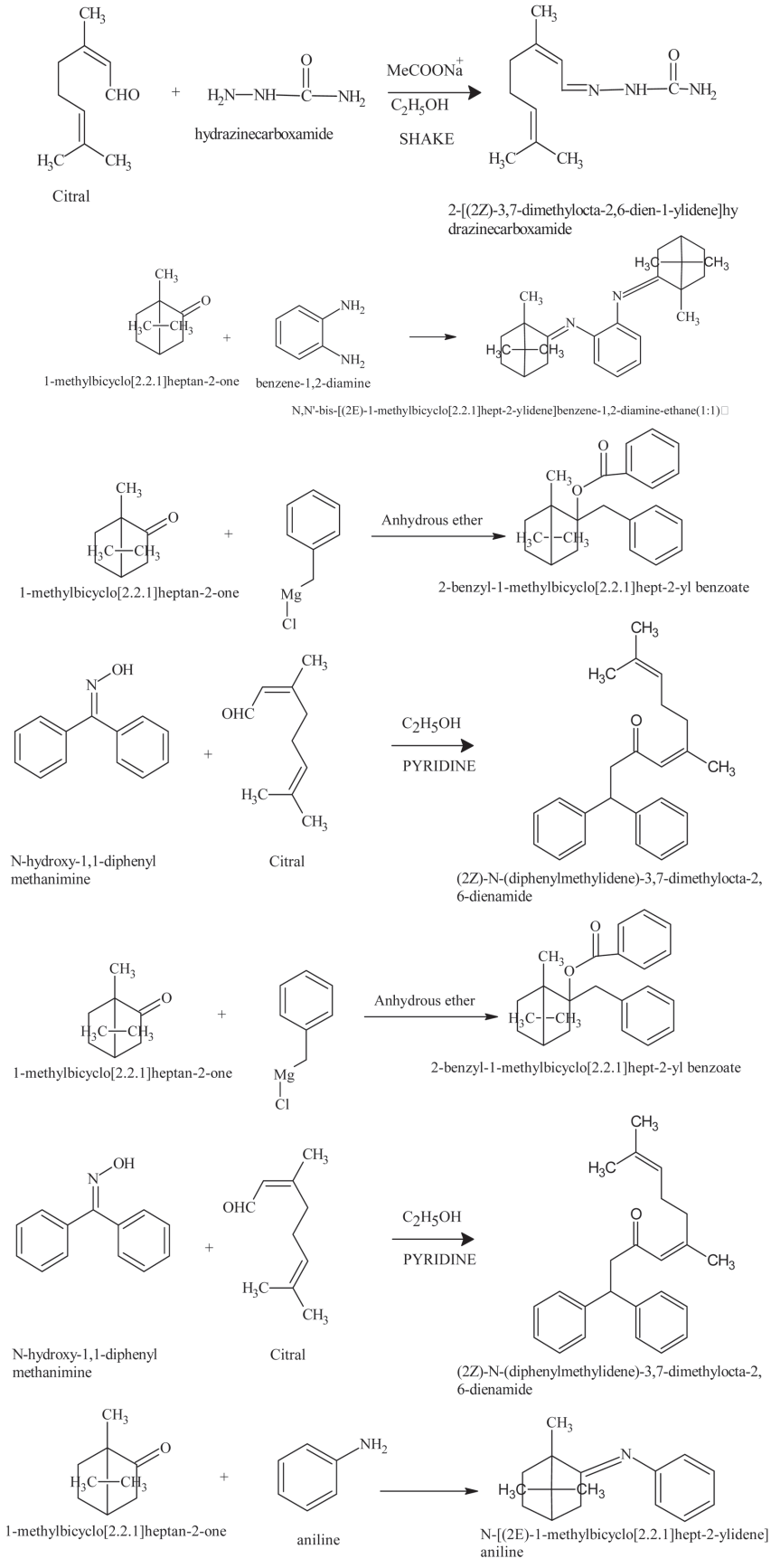
Antibacterial and antifungal activity of citral and camphor derivatives

Compound	Antibacterial Activity (Zone of inhibition in mm)				Antifungal Activity (Zone of inhibition in mm)			
	B. Subtillis	*S. Aureus*	*E. coli*	*P. vulgaris*	*A. niger*	*C. verticulata*	*F. oxysporum*	*A. flavus*
I	18	17	16	14	19	16	12	10
II	14	15	14	12	15	13	10	08
XVII	16	12	13	11	17	12	10	04
XVIII	15	13	12	13	14	13	08	09
XIX	16	11	14	12	14	12	10	03
Standard (10 µg/cup)	22	20	18	17	21	22	23	15

*Concentration of Test Compound: 100 µg/cup
Standard : Ampicillin

*Concentration of Test Compound:100 µg/cup
Standard: Clotrimazole

Fig.1: Scheme for the synthesis of citral and camphor derivatives



clotrimazole in 10 ml of dimethylformamide (AR grade). Further, the dilution was made with dimethylformamide itself to obtain a solution of 100 µg/ml concentration.

The potato-dextrose-agar medium was sterilized by autoclaving at 121°C (15 lb/sq. inches) for 15 minutes. The petriplates, tubes and flasks with cotton plugs were sterilized in hot-air oven at 150°C, for an hour. In each sterilized petriplate, about 27 ml of molten potato-dextrose-agar medium inoculated with respective fungus (6 ml of inoculum in 300 ml of potato-dextrose medium) was added aseptically. After solidification of the medium at room temperature three discs of 6 mm diameter were made in each plate with a sterile borer. Accurately 0.1 ml (100 µg/disc) of test solution was transferred to the discs aseptically and labeled, accordingly. The reference standard, 0.1ml (10 µg/disc) was also added to the discs in each plate. The plates were kept undisturbed at room temperature for 2 hours, at least to allow the solution to diffuse properly into the potato-dextrose-agar medium. Then the plates were incubated at 25°C for 48 hours. The diameter of the zone of inhibition was read with the help of an antibiotic zone reader. The experiments were performed in triplicate in order to minimize the errors. The results are presented in Table.No.1.

Result and Discussion

Antibacterial activity

The results represented in Table No.1. Proves that both the citral derivatives posses antibacterial activity. Among them compound II has shown better antimicrobial activity with a greater zone of inhibition of 18mm in comparison with 14mm of compound I. But when compared with the standard drug Ampicillin, compound II has shown moderate antibacterial activity. While comparing the structure of compound I and compound II, both the compounds contain Nitrogen hetero atom where as the compound II contains Keto group and the aromatic rings which are absent in compound I. This states that removal of keto group is essential for the antimicrobial activity, attaching the amine substituted aromatic ring to the aldehyde group will show better activity than attaching a nitrogen attached to a aliphatic compounds.

All the three camphor derivatives has shown mild to moderate antimicrobial activity, but none of the derivatives showed comparatively good antimicrobial activity in comparison with the standard drug (Ampicillin).

Antifungal activity

Citral derivatives were subjected for antifungal activity against *A.niger, C. verticulata, F. oxysporum, A. flavus and* compared with standard drug. Among them compound I showed better activity with zone of inhibition of 19mm against *A.niger* but was not comparatively active against other organisms.

Camphor derivatives showed mild to moderate activity against the organisms used for antifungal activity. Compound III has shown better activity when compared with the other derivatives of camphor. It has the zone of inhibition of 17mm, 12mm, 10mm and 04mm against A.niger*, C. verticulata, F. oxysporum, and A. flavus* respectively.

Conclusion

The study suggests that both citral and camphor derivatives possess moderate to good antibacterial activity but did not show promising antifungal activity. Thus it can be concluded that, further optimization of citral and camphor derivatives may lead to a potential antibacterial agent.

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Arvensin, A New Flavanoid Glycoside from Polygala Arvensis

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ABSTRACT

Polygala arvensis belonging to the family Polygalaceae is a source of variety of compounds having pharmacological activity and inexplored. In the present work we report the isolation and structural elucidation of a new flavonoid glycoside. The spectral data confirms the presence of Arvensin.

Keywords: Polygala arvensis, Phytochemical, Flavonoid glycoside.

Introduction

The family Polygalaceae consisting of ten genera and about 500 species of flowering plants commonly known as milkwort or snakewort. The genus include herbaceous perennial plants, shrubs and small trees and has cosmopolitan distribution except Newzealand and Artic regions. This family is characterised by a peculiar androecial structure, modified perianth and a biloculate ovary. The flowers superfically resemble to the papilonaceous flowers of Leguminoseae. They are used as food plants and as ornamental plants. The genus Polygala is a source of variety of compounds and extensively explored and are known source of pharmacologically important compounds. Hence it is felt worthwhile to take up chemical investigation of P.arvensis locally avaiable and untested. A detailed report of spectral and chemical methods adopted to deduce the structure form the content of present paper. P.arvensis is an annual prostrate or procumbent herb. The leaves are 1.3*5.1,obovate, suborbicular, apex emarginate. Flowers are yellow in axillary or extra axillary short almost capitate, four flowered raceme. The sepals unequal, outer smaller than inner wings, ovate or falcate, perberulous, acute. Petals 3,laterals broadally obovate, hooked, crest subapical, appendage, fimbricate. The stamens with 8 filaments united for their lower half into a split sheath another oblong porous, ovary obovoid, style incurved, apex hooked, stigma broad, capitate, capsule ovoid, flat. Emarginate margin, pubscent. The seeds are ovate or ellipsoid, villous, caruncle 3-lobed. The flowering is done during August to January. The phytochemical study of Polygala arvensis resulted in the isolation of a new flavanoid glycoside arvensin which is presented in this paper.

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Extraction And Isolation

The entire plant material was macerated in a blender with alcohol till no colour was imparted to the extract. The blended material was filtered and concetrated to 250ml under reduced pressure. In order to make it free from chlorophyll it was extracted with toluene. The resulting extract was extracted with ethyacetate. The ethylacetate extract was yellow in colour and on evaporation it gave a yellow solid compound-A. The left over aqueous extract was concentrated under reduced vaccum. The residue was triturated with ethylalcohol which was concentrated to 50 ml and left over for refrigeration for couple of days. A white shining crystalline compound was separated out. It was recrystallised from a mixture of alcohol and ether to get compound-B. The compound-A obtained from ethylacetate was found to be new flavonoid glycoside named arvensin.

Structural Study Of Compound

The pale yellow coloured crystalline compound was analysed for its elemental contenets and gave $C_{21}H_{20}O_{12}$. It melted at 269-271 ^{o}C and was optically active. It gave a pink colour when treated with Mg and HCl which indicated flavanoid compound and dark green colour with neutral ferric chloride indicating presence of a free phenolic group. Molisch test was positive therefore the compound was glycosidal in nature. It was tested for homogenecity using paper chromatography (Rf-0.38, n-buOH-AcOH-H_2SO_4, 4:1.5:0.3 in 5% AcOH). It gave a yellow colour with green fluoroscene on treatment with conc. H_2SO_4 and bright green colour with green fluoroscene on treatment with aqueous NaOH. The physcial data of the compound indicated it to be a new flavanoid glycoside and named it arvensis which was suported by spectral data.

Spectral Analysis

The light absorption of arvensis appeared at 242,259,317 and365nm. The range of absorption confirmed its flavanoid nature. The following conclusions were drawn from the U.V. spectral analysis with the shift reagents. The presence of hydroxyl group at 4 and 5, thereby no substitution at the 7-hydroxyl group and the presence of O-dihydroxy function in B-ring.

IR spectral data

The IR spectral studies suggested the presence of hydroxyl group, carbonyl and aromatic double bonds.

V_{max} Nujol: 3350, 2800, 1640, 1620, 1595, 1480, 1400, 1295, 1240, 1180, 1040, 980.940.760 cm-1.

PMR spectral data

The peak values are singlet (6.35), singlet (6.79), singlet (6.81), singlet (7.38), broadsinglet (8.15), singlet (13.75), doublet (4.63), multiplet (3.3-3.9), broad multiplet (3.9-4.2). Thus the resonance observed in PMR are in complete agreement with proposed structure.

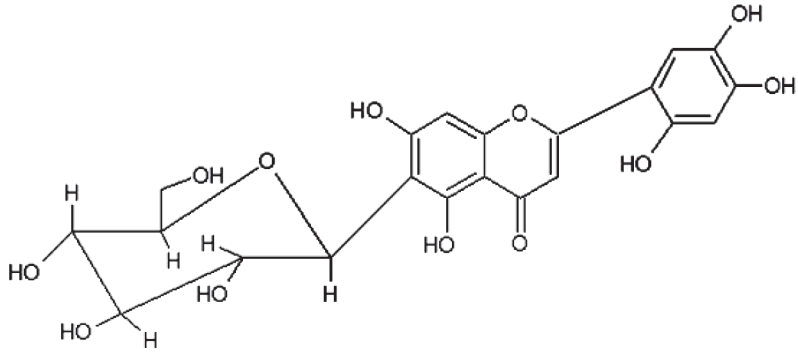
^{13}C-NMR spectral data in DMSO-d_6

The chemical shift values are furnished along with their assignment.

179.03(4), 163.67(7), 161.67(2), 161.33(5), 156.25(9), 101.31(3), 108.04(6), 102.49(10), 93.35(8), 154.04(4'), 150.84(5'), 143.62(2'), 111.67(1'), 107.95(3'), 107.41(6'), 81028(5''), 78.80(3''), 73.10(1''), 70.48(2''), 70.31(4''), 61.34(6'').

Mass spectral data

The signals that appeared in the mass spectrum of the compound are given here under with relative intensities. m/z:164(M+), 146(M+H_2O), 133(M+CH_2OH,base peak), 115, 103, 87, 73, 69, 61, 60 and 57. The above data confirms the following structure of the new flavanoid glycoside arvensin.



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