SYNTHESIS ,CHARACTERIZATION AND ANTIMICROBIAL ACTVITY OF NOVEL ANALOGUES OF FLAVONES

B.S.Jayashree[a], Abhishekh Kumar[a], Aravinda Pai[b], Yogendra Nayak[b]

Department of Pharmaceutical chemistry, Manipal college of Pharmaceutical Sciences, Manipal

Department Of Pharmacology, Manipal college of Pharmaceutical Sciences, Manipal

Manipal University

Summary

In the present work a series of novel flavone analogues have been synthesized, characterized by various spectrochemical methods. All the compounds have been evaluated for their antioxidant potency, and screened for antimicrobial activity . Out of the 10 compound synthesized the compounds JAK4 and JAK5 have shown antibacterial activity.

Introduction

Flavonoids are ‘’the most common group of polyphenolic compounds in the human diet and are found ubiquituously in plants’’. Flavanoids are most commonly known for their antioxidant properties. Flavonoids having benzopyrone moiety are known to exhibit antimicrobial, antihypertensive, antiplatelet, gastroprotective, antitumor, antiallergic and antidiabetic activies. They produce such biological effects through their free radical scavenging antioxidant activities and metal ion chelating abilities[7]. It has also been screened for their antibacterial activity.

Literature review of the compounds having benzopyrone moiety and the various chemical modifications on benzopyrones supported different biological activities such as antibacterial, antihypertensive, anticholesteromic, antiinflamatory and hypoglycaemic activities.

Materials and Methods

A Synthesis of Chalcone

Procedure

To a solution of 0.05 mole of 15ml of o-hydroxy acetophenone in 76 ml of alcohol and 15.5 ml of 50% potassium hydroxide was added, 0.06 mol of 17.8g of p-dimethyl amino benzaldehyde and the mixture was refluxed on water bath for 1 hr. and left overnight. The deep red solution formed was poured into crushed ice and acidified with hydrochloric acid. The reddish brown precipitate was then separated by filtration and recrystallised from acetone.

Preparation of 3-hydroxy flavone

Procedure

To a suspension of 0.01 ml of chalcone in 85 ml of ethanol was added l0ml of 20% aqueous sodium hydroxide with stirring, followed by the careful addition of I8 ml of 30% hydrogen peroxide over a period of 0.5 hrs. The reaction mixture was then stirred for 3.5 hrs at 30°C and was poured into crushed ice containing 5.0 N hydrochloric acid. The precipitate obtained was filtered, washed, dried and recrystallised from ethyl acetate.

Synthesis of 3-Benzoyl-7-hydroxy-6-nitroflavone[64]

Procedure

27g of anhydrous potassium carbonate was added to a stirred solution of 5.30g of 2',4'-dihydroxy-5'-nitroacetophenone in 300ml of dry acetone.The mixture was stirred at room temperature for 10 minutes and then 6.3 ml of benzoyl chloride was added dropwise and the mixture was then stirred at room temperature for an additional 30 minutes.After refluxing for 24 hours , the solvent was evaporated under reduced pressure. The residue was cooled to room temperature and acidified in a beaker with dilute hydrochloric acid just to make it acidic.The precipitate formed was then filtered,dried and recrystallised from glacial acetic acid.

Synthesis of 3-Benzoyl-7-hydroxy-6-aminoflavone

Procedure

A solution of 1.4g of sodium hydrosulfite in 10 ml water was added rapidly to a stirred suspension of 0.83g of 3-Benzoyl-7-hydroxy-6-nitro flavone in 6ml of 30% aqueous ammonia. The reaction mixture was warmed spontaneously and as the suspended solid began to dissolve an orange coloured precipitate appeared. Further it was boiled for 15 minutes and was cooled to room temperature and product was recovered by filtration.

PHYSICO CHEMICAL PARAMETERS

Partition coefficient

The hydrophobic character of a drug can be measured experimentally by testing the drug's relative distribution in an octanol/water mixture.[66]Hydrophobic molecules will prefer to dissolve in the octanol layer of this two-phase system, whereas, hydrophilic molecules will prefer the aqueous layer. The relative distribution is known as partition coefficient denoted by **P** and is obtained from the following equation:

$$P = \frac{\text{Concentration of drug in Octanol}}{\text{Concentration of drug in aqueous layer}}$$

Hydrophobic compounds will have a high P value, whereas, hydrophilic compounds will have a low P value.Various substituents on the lead compound will produce a series of analogues having different hydrophobicities and different P values. By plotting the P values against the biological activity of these drugs, it is possible to see if there is any relationship between the two drugs. The biological activity is normally expressed as 1/C, where C is the concentration of drug required to achieve a defined level of biological activity.

- The organic phases used for studying partition coefficient include alkanes, chloroform, hydrogen bond donor, n-octanol amphiprotic and propylene glycol dipelargonate. Among these, n-octanol is the solvent of choice because of the following advantages over other solvents.

The various hydrophobic parameters include:
(1)Partition coefficient(P, log P)
(2)Substituent hydrophobicity constant(π)
(3)Hammett substituent constant

In the present study, shake flask method is employed using n-octanol and water system.

Shake flask method
Principle

In order to determine partition coefficient, equilibrium between all interacting components of the system must be achieved, and the concentration of the substances dissolved in two phases must be determined. A study of the literature on this subject indicates that several different techniques can be used to solve this problem namely, the thorough mixing of the two phases followed by their separation in order to determine the equilibrium concentration for the substance being examined.

The measuring range of the method is determined by the limit of detection of the analytical procedure. This should permit the assessment of values of log P in the range of 2 to 4, occasionally when conditions apply, this range may be extended to log P up to 5, when the concentration of the solute in either phase is not more than 0.01 mole per liter.

Experimental

Requirements**:** Conical flasks, Test tubes, Separating funnel, n-Octanol AR gr

Procedure followed

The λ_{max} for the test compounds in n-octanol was determined and the absorbance at that wavelength was noted (B_E-Absorbance before extraction). 20 ml of the10µg/ml drug solution in n-octanol and 20 ml of water was added to 250 ml conical flask, covered with parafilm and shaken in the mechanical shaker for 24 hours to create an endogenous environment. The phases were then kept without any disturbances for proper separation. The absorbance of the separated n-octanol phase was then measured at the λ_{max} specific for that compound. (A_E- Absorbance after extraction).

$$P = C_{organic}/C_{aqueous}$$

$$= B_E / B_E - A_E$$

The Log P was then calculated, which gives an idea about the solubility and lipid profile of the test compound.

BIOLOGICAL ACTIVITY

Antioxidant activity

Antioxidants are molecules that can neutralize free radicals by accepting or donating an electron to eliminate the unpaired condition. Free radicals are molecules produced when our body breaks down food, or by environmental exposures like tobacco smoke and radiation. Free radicals can damage cells, and may play a role in heart disease, cancer and other diseases. Certain antioxidant enzymes are produced within the body. The most commonly recognized of these naturally occurring antioxidants are Superoxide Dismutase, Catalase, and Glutathione. Superoxide Dismutase changes the structure of oxidants and breaks them down into hydrogen peroxide. Catalase in turn, breaks down hydrogen peroxide into water and tiny oxygen particles or gases. Glutathione is a detoxifying agent, which binds with different toxins to change their form so that they are able to leave the body as waste

Measurement of Antioxidant activity

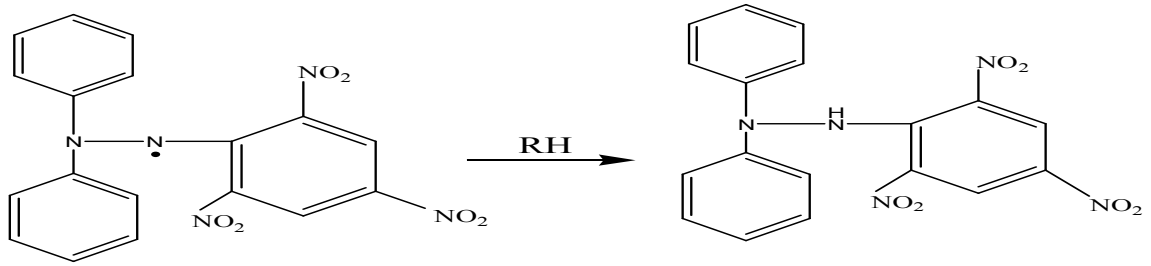
A wide range of antioxidants both synthetic and natural have been proposed in the treatment of human diseases. Hence, considerable attention has been devoted for the development of techniques for measurement of antioxidant activity. A number of methods are available for screening of antioxidants including *in vitro* and *in vivo* methods.

- Measuring the ability to donate an electron or hydrogen atom to a specific reactive oxygen species or to any electron acceptor.

- Testing the ability to remove any source of oxidative initiation e.g. inhibition of enzymes, chelation of transition metal ions and absorption of UV radiation.

The *in vitro* methods include conjugated diene assay, DPPH· method, inhibition of super oxide radical formation, hydroxyl radical scavenging activity, nitric oxide radical inhibition activity, ABTS method etc. The *in vivo* models include microsomal lipid peroxidation and erythrocyte ghost system.

DPPH Method .



Procedure

- **DPPH· solution**

The DPPH· stock solution was prepared by dissolving 39 mg of the DPPH· in 50 ml of methanol.

- **Standard solution**

Ascorbic acid was used a standard free radical scavenger. This was prepared by dissolving 1mg of ascorbic acid in 10 ml of methanol to get a 100µg/ml stock solution.Serial dilutions were then made so as to get concentrations of 50 µg/ml, 25µg/ml, 12.5µg/ml and 6.25µg/ml each.

- **Test solution**

The test solutions of the test compounds were prepared in methanol, by dissolving 1 mg of the test compound in 1ml methanol to get 1000µg/ml stock solution. Serial dilutions were then made so as to get concentrations of 500µg/ml, 250µg/ml, 125µg/ml, 61.25µg/ml, 30.63µg/ml, 15.3µg/ml and 7.65µg/ml each.

- **Method**

The assay was carried out in a 96 well microtitre plate. To 100µl of methanol solution, 100µl of each of the test sample or the standard solution was added separately in wells of the microtitre plate in triplicate. Control was prepared by adding 100µl methanol in 100µl DPPH· solution. The plates were incubated at 37˚C for 20 minutes and the absorbance of each solution was measured at 540nm using ELISA reader against the corresponding test and standard blanks and the remaining DPPH· was calculated.IC_{50} is the concentration of the sample required to scavenge 50 % of DPPH· free radicals.

$$\% \text{ inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

a. Nitric oxide radical inhibition assay

Chemicals and Reagents

1. Sodium nitroprusside solution: 0.2998g of sodium nitroprusside was dissolved in 100ml of distilled water.
2. Naphthyl ethylene diamine dihydrochloride: 0.1% of the solution was made up with 20% glacial acetic acid and made up the volume to 100ml.
3. Phosphate buffer saline,pH adjusted to 7.4
4. DMSO distilled

Method

600 µl of the reaction mixture containing 400µl sodium nitroprusside, 100µl of phosphate buffer, 100µl of drug solution of different concentrations were taken in a microcentrifuge tube and incubated at 25˚C for 150 minutes. After incubation to 50µl of the reaction mixture ,100µl of sulphanilic acid was added in microtitre plate. It was mixed well and allowed to stand for diazotisation for 5 minutes .To this 100µl of Naphthyl ethylene diamine dihydrochloride was added and allowed to stand for 30 minutes in diffused light at room temperature. The absorbance was measured at 540 nm using ELISA reader against

corresponding blank solution.IC_{50} is the concentration of the sample required to inhibit 50% of nitric oxide radical.

E Antibacterial activity

Agar diffusion method

In this technique, melted agar inoculated with microorganisms is poured into petridishes. Wells are made in the agar plate and a specific volume of the antimicrobial substances are placed in them, plates were incubated at a temperature of 37^{o}C for the respective time. The antimicrobial substance diffuses through agar around its well and produces a clear zone of inhibition. The diameter of this zone gives an estimation of the degree of activity of the antimicrobial substance.

Tube dilution method

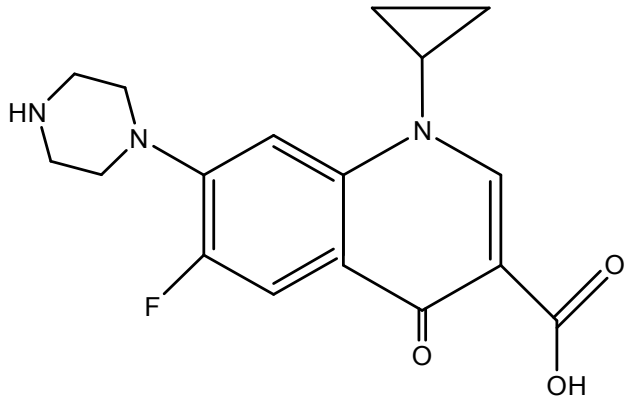
This method is used for determining the antimicrobial susceptibility in liquid media. The dilutions of the antimicrobial agents are prepared in growth medium such that the concentration of the drug covers its clinical significant range. An equal volume of broth containing 10^{5}-10^{6} bacteria/ml is added to each tube and to a control tube without any antimicrobial agent. The tubes are examined for visible turbidity after overnight incubation at 37^{o}C. The decrease in the turbidity gives the antimicrobial activity.

In the present study, an attempt has been made to perform the qualitative antibacterial activity by agar diffusion method.

Antibiotics used as standards in the present work

Ciprofloxacin

Ciprofloxacin is a 6-fluoro quinolone class of antibiotics, acting on bacterial DNA gyrase subunit A and having a broad spectrum of action. It is the agent of choice for the treatment of many bacterial infections including chronic infections.



Experimental
Agar diffusion method
Requirements: Petridishes, corkborers, micro pipette ,swabs,microtips

Procedure

Stock solutions of synthesized test compounds and standard drugs were prepared in DMSO. The test compounds were used at 250µg,125µg,62.5µg and 31.25µg/ 50µl .
Ciprofloxacin was used as the standard at 25µg/50µl.

Microorganisms used

Standard cultures of *Bacillus subtilus, Staphylococcus aureus* (Gram-positive) and *Escherichia coli, Pseudomonas aeruginosa* (Gram-negative) were used. These were obtained from the Department of Pharmaceutical Biotechnology, MCOPS. The microorganisms were maintained by sub-culturing and used at regular intervals in Mueller-Hinton agar medium.

Medium used: Mueller-Hinton agar

The ingredients of the medium are as follows

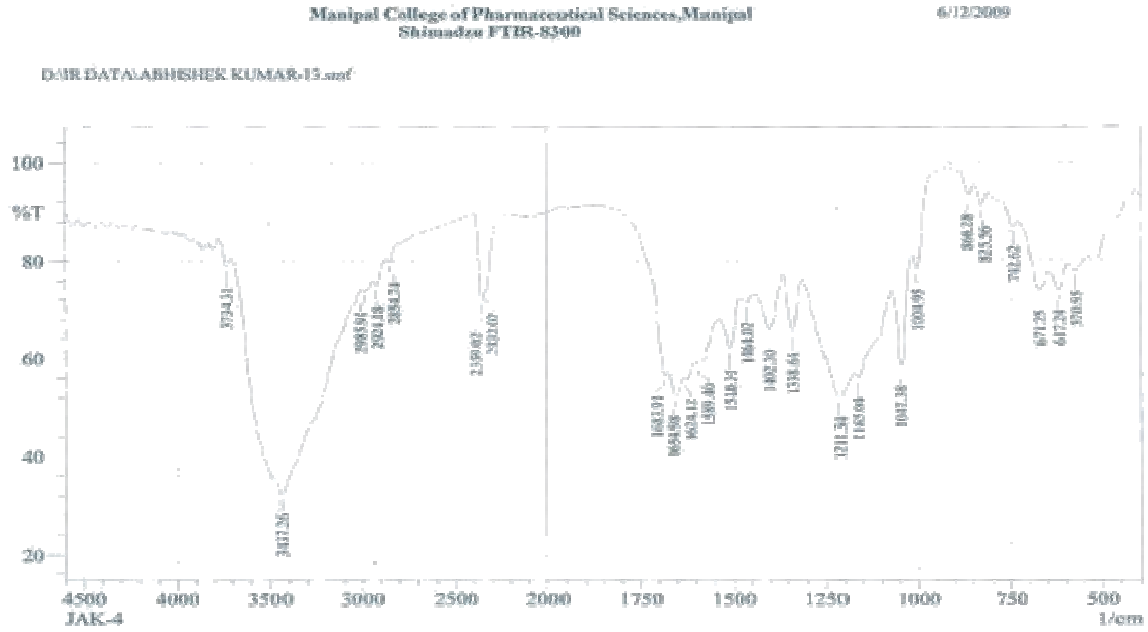
Ingredients	Concentration(g/l)
Beef infusion	300
Casein hydrolysate	17.5
Starch	1.5
Agar	17
Final pH at 25˚C	7.4+0.2

About 38 g of the above medium with 3g of agar was suspended in 1000ml of distilled water in a conical flask, corked with non-absorbent cotton and sterilized by autoclaving at 15 lbs pressure, at 121^{o}C for 15 minutes.

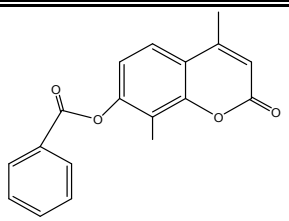
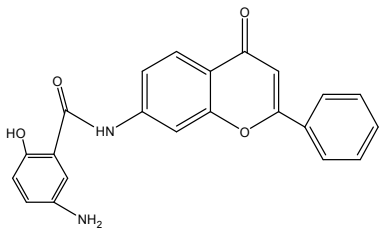
The petridishes were thoroughly washed and sterilized in hot air oven at 160^{o}C for one hour. The inoculum was added to the medium and was poured into sterile petridishes for solidification. Wells were made on the medium using sterile borer after solidification. 50µl of the test and standard solutions were added to the respective bores. A control having only DMSO was maintained in each plate as control.

The petridishes were kept in the refrigerator for 20 minutes for diffusion to take place and then incubated at 37^{o}C for 24 hours; the zone of inhibition was observed and measured using a scale. The antibacterial activity of the test compounds was carried out against four microorganisms.

IR SPECTRA OF ACTIVE COMPOUND JAK 4



Results and Discussions

Sr. No.	Compound Code	Structure	λmax	P	Log P
1	JAK-1		297.4	83.17	1.92
2	JAK-3		281.60	53.70	1.73
3	JAK-4		257	2.29	0.36

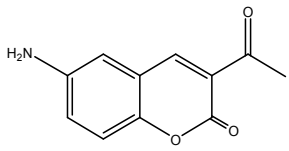
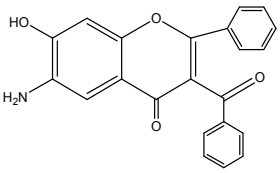
					
4	JAK-6		260	41.69	1.62

Table 9 showing DPPH· radical scavenging

Conc µg/ml	**JAK 4**	**JAK 5**
3.9	42.6	2
7.8	54.43	3.28
15.63	77.13	15.75
31.25	93.62	18.21
62.5	90.96	19.49
125	89.36	21.13
250	87.59	21.68
500	85.46	21.49
IC_{50}	**215**	**-**

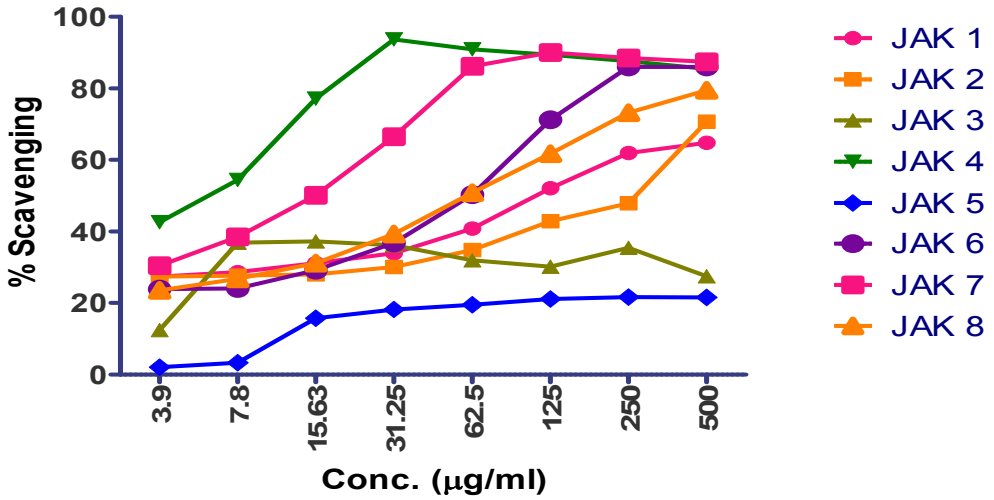


Table 11 showing the antibacterial activity of the synthesised test compounds

Sr. No	Comp. code	*B.subtilis*		*S.aureus*		*P.aureginosa*		*E.coli*	
		Zone (mm)	Conc (µg/50µl)	Zone (mm)	Conc (µg/50 µl	Zone (mm)	Conc (µg/50µl)	Zone (mm)	Conc (µg/50µl)
1	JAK 4	28 25 20	250 125 62.5	20 18	250 125	-	-	-	-
2	JAK 5	23 20 18 14	250 125 62.5 31.25	15 13	250 125	-	-	19 16 15	250 125 62.5

Summary And Conclusion

- The parent test compounds flavones were synthesised.
- Yields of the intermediates before arriving at the final test compounds were found to be in the range of 36-86%.
- All the test compounds were purified by recrystallisation using solvents ,such as ethanol, methanol, acetone, ethyl acetate and glacial acetic acid and the purity was checked by melting point determination and TLC.
- All the purified test compounds including their parents were characterised by spectral studies by using UV, IR, ^{1}H NMR and Mass spectra to support their structures.
- Yields of the final test compounds were obtained in the range of 68 to 91%.

- The IR spectra of the test compounds were characterised by the presence of specific functional groups present in the molecule.
- The ^{1}H NMR spectra of the test compounds were characterised and supported their structures.
- The mass spectra of the test compounds were characterised by the presence of molecular ion peak and its fragments.
- Log P values obtained for the test compounds , were found to be in the range of 0.36 to 1.92.
- The antioxidant activity of the test compounds were done using DPPH radical scavenging activity and Nitric oxide scavenging activity.
- The antibacterial activity of the test compounds were performed against four species of bacteria namely *Bacillus subtilis*, *Staphylococcus aureus* (Gram positive) and *Pseudomonas aureginosa* and *Escherichia coli* (Gram negative) .

Conclusion

- Yields of the intermediates and the final test compounds obtained were found to be in the range of 36-86% and 68 to 91% respectively .
- 2 compounds, incuding their parents and final products are totally new and the procedure was standardised to improve the yield and all the physical constants were fixed for them.
- Out of the 10 test compounds tested for their DPPH radical scavenging activity JAK 4 showed antioxidant activity . Out of the eight test compounds tested for their Nitric oxide radical scavenging activity, 1 test compounds , JAK 4 showed antioxidant activity below 100µg/ml when compared to that of standard Ascorbic acid at IC_{50} value of 4.73 µg/ml.

  Out of the eight test compounds tested for their antibacterial activity, 2 test compounds JAK 4 and JAK 5 showed zone of inhibition at 31.25 to 250 µg/50µl comparable to that of the standard ciprofloxacin 25 µg/50µl .

References

1. John AO Ojewole . Hypoglycaemic effect of Clausena anisata(Wild) Hook methanolic root extract in rats. Journal of Ethnopharmacology 2002; 81(2) : 231-237.
2. Simone M de Souza et.al . Antibacterial activity of coumarins. Z Naturforsch. 2005; 60c: 693-700
3. Vibhute Y. B. Synthesis and antimicrobial activity of some halo-chalcones and flavones. Journal of the Indian Chemical Society.1976; 53(7): 736-8
4. Creuzet MH et.al . 3',4',5,7-Tetrahydroxyflavone derivatives and their therapeutical use . Eur. Pat. Appl. 1984
5. Hogale M B , Pawar B N and Nikam B P. Synthesis and biological activity of some new flavones Journal of the Indian Chemical Society.1987; 64(8): 486-7.
6. Tang L , Zhang S , Yang J , Gao W , Cui J and Zhuang T. A novel approach to the synthesis of 6-amino-7-hydroxy-flavone . Molecules . 2004 ; 9-842-848 .
7. Graham L.Patrick . An introduction to Medicinal Chemistry. 1995;
8. Tripathi KD . Essentials of Medical Pharmacology. 2003; 5:630
9. Sharma YR . Elementary Organic Spectroscopy, Principles and Chemical applications . 2005; 179-181