SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL ACTVITY OF NOVEL ANALOGUES OF FLAVONES

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Summary

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

Introduction

Flavonoids are ''the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants''. They have a benzopyrone moiety and are known to exhibit antimicrobial, antihypertensive, antiplatelet, gastroprotective, antitumor, antiallergic and antidiabetic activies. They produce such biological effects owing to their free radical scavenging activities and metal ion chelating abilities. Free radicals are produced when our body breaks down food, or by environmental exposures like tobacco smoke and radiation can damage the 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 are the naturally occurring ones such as, 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.

Materials and Methods

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 recrystallized 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 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 recrystallized 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 5.30 g 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 drop wise 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. The precipitate formed was filtered, dried and recrystallized 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 into 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.

Partition coefficient study:

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:

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

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

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

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 the 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 as a standard free radical scavenger. This was prepared by dissolving 1 mg 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.

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

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 micro centrifuge tube and incubated at 25˚C for 150 minutes. After incubation to 50µl of the reaction mixture, 100µl of sulphanilic acid was added into microtitre plate. It was mixed well and allowed to stand for diazotization for 5 minutes .To this, 100µl of naphthyl ethylene diamine hydrochloride 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 and their IC_{50} was calculated which 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 was poured into petridishes. Wells were made in the agar plate and a specific volume of the antimicrobial substances were placed in them, plates were incubated at a temperature of $37^{o}C$. The antimicrobial substance after diffusing through agar 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 were prepared in growth medium such that the concentration of the drug covers its clinical significant range. An equal volume of broth containing $1\text{-}10^{6}$ bacteria/ml was added to each tube and to a control tube without any antimicrobial agent. The tubes were 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, micro tips

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 subtilis, 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

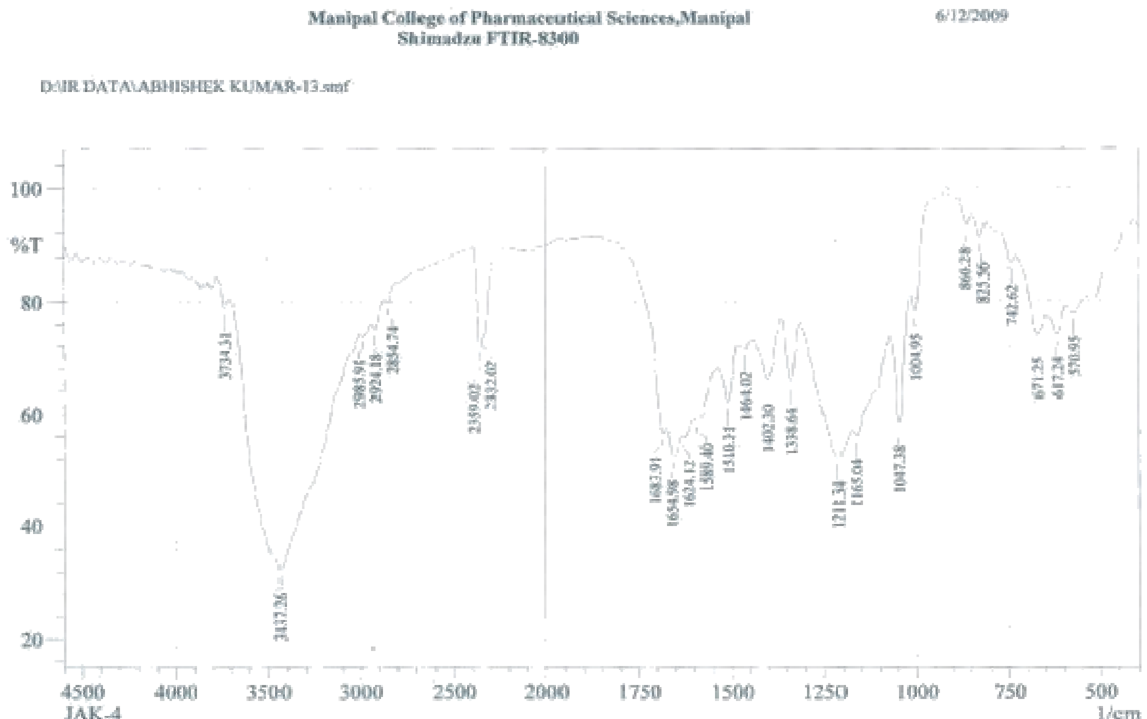
Ingredients	Concentration(g/l)
Beef infusion	300
Casein hydrolysate	17.5
Starch	1.5
Agar	17
Final pH at 25˚C	7.4+0.2

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°C for 15 minutes.

The petridishes were thoroughly washed and sterilized in hot air oven at 160°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°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**	**-**

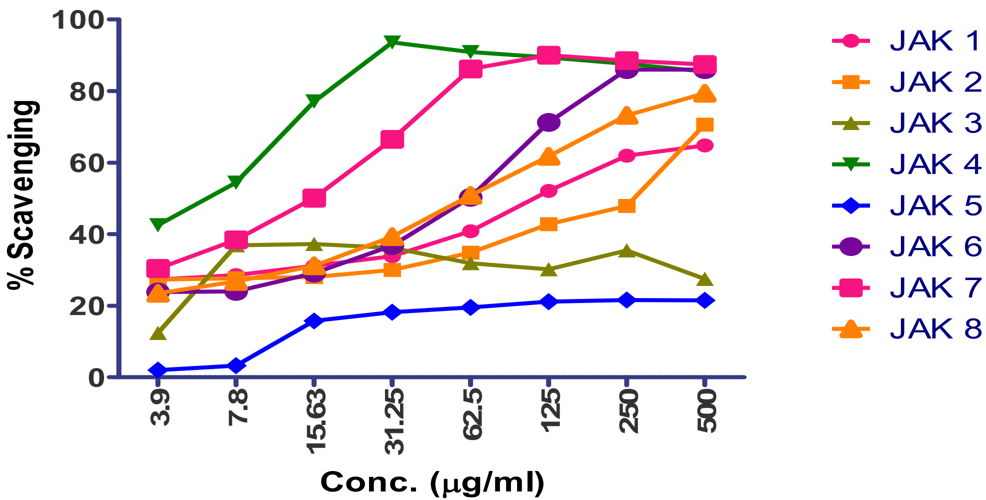


Table 11 showing the antibacterial activity of the synthesised test compounds

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

- 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 recrystallization 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 characterized 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 characterized by the presence of specific functional groups present in the molecule.

- The ^{1}H NMR spectra of the test compounds were characterized and supported their structures.
- The mass spectra of the test compounds were characterized 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, including their parents and final products are totally new and the procedure was standardized 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.

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