SYNTHESIS OF SUBSTITUTED 3-HYDROXY FLAVONES FOR ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY

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Summary

Flavonoids are phenolic compounds widely distributed in the plant kingdom. They are known to exhibit antioxidant, anti-inflammatory, antimicrobial, antihypertensive, antiplatelet, gastroprotective, antitumor, and antiallergic activies. Many available literatures prompted us to modify the benzopyrone ring present in the flavonoids to explore newer activities associated with this nucleus. The chalcones were prepared by reacting o-hydroxy acetophenone with dimethylamino benzaldehyde. 3-hydroxy flavone was prepared by reacting chalcone with alkaline hydrogen peroxide. 3-methoxy and 3- acetoxy flavone were prepared by reacting 3- hydroxyl flavone with dimethyl sulphate in acetone medium and acetic anhydride respectively. The different esters of 3-hydroxy flavones were prepared by treating the 3-hydroxy flavone with different acid chlorides in the presence of pyridine. The test compounds were characterised by UV, IR, ^{1}H-NMR and Mass spectral studies. For each test compounds λ_{max}, ϵ_{max} and pKa were determined using standard protocol. Two out of twelve test compounds showed antibacterial activity against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa,* comparable to standard antibiotics Amoxicillin and Gentamycin. However, the test compounds found to have poorer DPPH· radical scavenging activity.

Key words: Antibacterial, Antioxidant, Chalcone, Flavones

Introduction

Flavonoids are phenolic compounds widely distributed in plants. Most of the flavonoids studied are polyhydroxylated derivatives which appear to selectively react with free radicals or systems related to the induction of inflammatory processes [1-3]. Flavonoids not only protect the oxidative stress but also have multiple targets in the inflammatory process [3]. They have shown to inhibit protein kinase C (PKC), protein tyrosine kinase (PTK) and mitogen activated protein kinase (MAPK) pathways there by it blocking the expression of cyclooxygenase (COX), Nitric oxide synthase (NOS), tumour necrosis factor alfa (TNF α), interleukin 1 beta (IL-1β) and interlekin-6 (IL-6). Numbers of flavonoids are found to inhibit the synthesis of the inflammatory mediators such as prostaglantins and leukotrienes, by directly inhibiting Phospholipase 2 (PLA_2) and / or also by indirect inhibition of PLA2 expression from the inflammatory signals.

Flavonoids having benzopyrone moiety are known to exhibit antimicrobial, antihypertensive, antiplatelet, gastroprotective, antitumor, antiallergic and antidiabetic activies [4-11]. Quercetin and related flavonoids are known to inhibit the growth of tumor cells and potentiates the cytotoxicity of DNA damaging anti-cancer drugs such as cisplatin. The discovery that a variety of flavonoids were protein-tyrosine kinases and angiogenesis inhibitors generated considerable interest in the structural activity relationship [12]. The protein-tyrosine kinase is thought to play a key role in mediating signal transduction from the CD_4 receptor during lymphocyte activation and is found to elevate in certain murine lymphomas and human colon carcinomas. Further *in vivo* studies are necessary to develop flavonoid-based anticancer strategies

Flavonoids possess antifungal, antiviral and antibacterial activity. Several groups of researchers have demonstrated synergy between active flavonoids as well as between flavonoids and existing chemotherapeutics. The antibacterial mechanisms of action of selected flavonoids have been proved and elucidated [5]. The activity of quercetin (3, 3', 4', 5, 7-pentahydroxy flavone), for example, has been at least partially attributed to the inhibition of DNA gyrase. It has also been proposed that sophoraflavone G and (-)-epigallocatechin gallate inhibit cytoplasmic membrane function, and that licochalcones A and C inhibit energy metabolism. Cushnie *et al.*,[5] have discussed different flavonoids whose mechanisms of action have been investigated including robinetin, myricetin, apigenin, rutin, galangin, 2,4,2'-trihydroxy-5'-methylchalcone and lonchocarpol A. These compounds represent novel leads, and future studies may allow the development of a pharmacologically acceptable antimicrobial class of agents.

Even though, natural flavonoids are highly potent, they show certain limitations as far as their stability, solubility characteristics and kinetics are concerned. Hence, with the knowledge of designing, flavonoids could be synthesized in the laboratory by convenient and cost effective methods. These vast literatures prompted us to modify the benzopyrone ring to explore the biological activities associated with this nucleus [13-15]. In the present work 3 hydroxy flavone derivatives are synthesized and explored for their antioxidant anti-inflammatory activities.

Materials and methods

Chemicals

The chemicals used were of AR grade and LR grade, purchased from Loba Chemicals, Qualigens, NR Chemicals, Lancaster, Sigma, Reachem, S.D Fine Chemicals Ltd., Merck and Hi-Media.

Experimental

Melting points of the test compounds synthesised were determined using Thiele's melting point apparatus) and were found uncorrected. The purity of the compounds was checked by thin layer chromatography (TLC) using silica gel G as stationary phase and combination of ethyl acetate, cyclohexane used as mobile phase. The spots resolved were visualized using iodine chamber. The UV - Visible Spectra was recorded in the range of 200 to 800 nm on UV -Visible spectrophotometer (Model Shimadzu 1601). The Absorbance was taken at the λ_{max} characteristic for each test compound and the wavelengths are recorded in nm (λ_{max}).

The IR spectra were recorded on a Fourier Transform IR Spectrometer (Model Shimadzu 8700) using KBr disc method. ^{1}H NMR (400MHz) spectra were recorded on AMX-400 liquid state NMR spectrometer (Indian Institute of Science, Bangalore) in $CDCl_3$ using tetramethylsilane as an internal standard. Mass Spectra was recorded on Sciex-3000 (Applied BioSystems, Canada) LC-MS-MS, "Triple Quadruple MS" using Electro spray Ionization Positive ion mode (Indian Institute of Science, Bangalore).

Synthesis

Synthesis of Chalcone

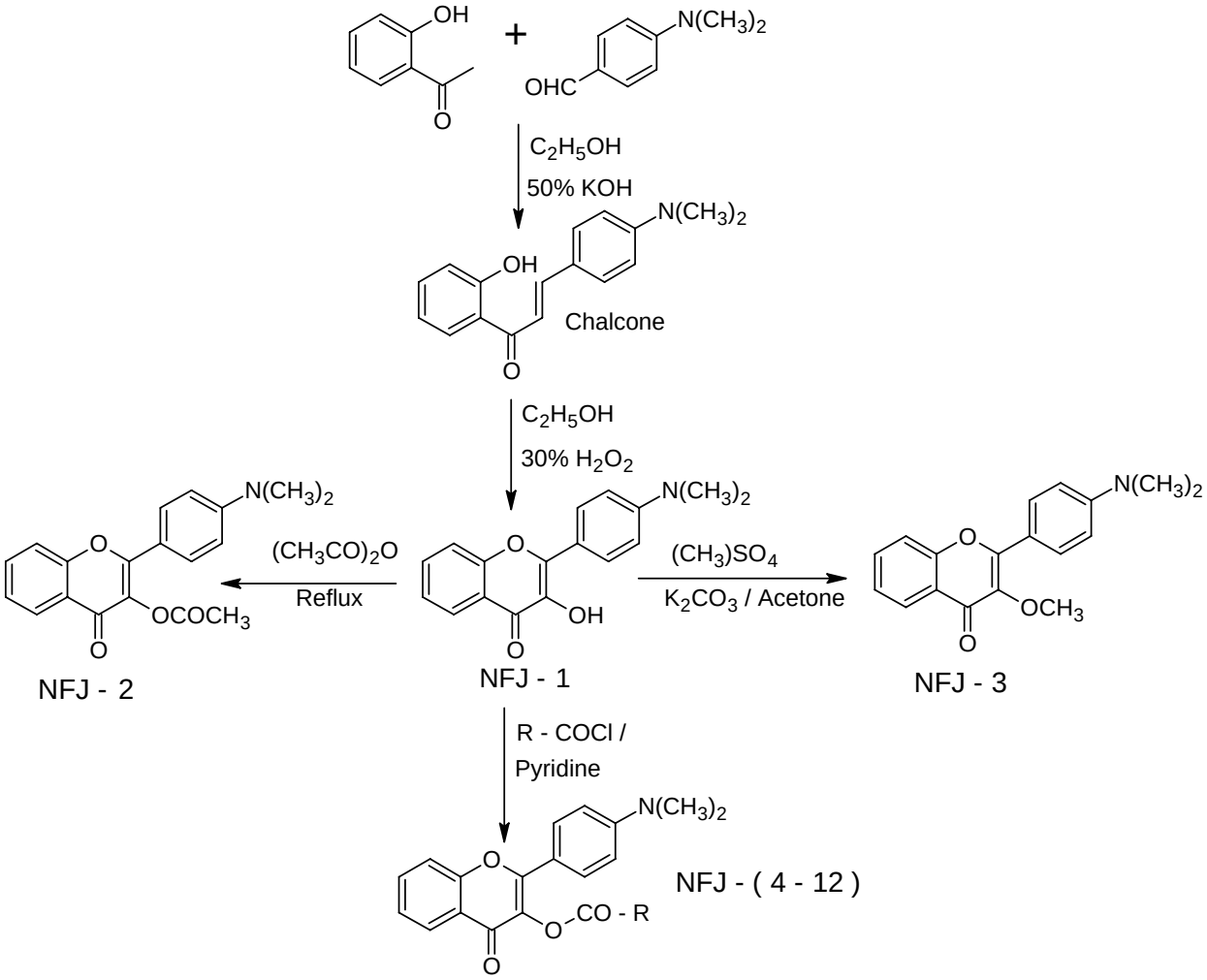
To a solution of 0.1 moles of o-hydroxy acetophenone in 152 ml alcohol and 31 ml of 50% potassium hydroxide, 0.12moles of p-dimethyl amino benzaldehyde was added and the mixture was refluxed on water bath for 1 hr. and left overnight. The deep red solution was poured into crushed ice and acidified with hydrochloric acid. The reddish brown precipitate was then separated by filtration and recrystallised from acetone.

Preparation of 3-hydroxy flavone

To a suspension of 0.01ml 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 hours. The reaction mixture was stirred for 3.5 hours at 30°C and was poured into crushed ice containing 5 N hydrochloric acid. The precipitate was filtered, washed, dried and recrystallised from ethyl acetate.

Preparation of 3-acetoxy flavone

To 0.01 moles of 3-hydroxy flavone in 100ml round bottom flask 10-15ml of acetic anhydride added and the mixture was refluxed for 2 hours. The resulting solution was cooled to room temperature followed by the addition of ice cold water. The solid separated was filtered, washed with cold water and recrystallised from ethanol.



Compounds	R	Compounds	R
NFJ-4	Cl	NFJ-9	CH_3
NFJ-5	Cl	NFJ-10	OCH_3
NFJ-6	NO_2	NFJ-11	Br
NFJ-7	NO_2	NFJ-12	
NFJ-8	CH_3		

Scheme 1: Synthesis of 3- Hydroxy Flavone Derivatives

Preparation of 3-methoxy flavone

0.01 moles of 3-hydroxy flavone was suspended in dry acetone containing powdered anhydrous potassium carbonate (0.03moles) and dimethyl sulphate (0.02moles). The suspension was refluxed for 5 hours. The solvent was evaporated under pressure and the residue diluted with water. The precipitate obtained was filtered, washed, dried and recrystallised from Ethanol.

Preparation of substituted acid chlorides

0.004 moles of substituted carboxylic acids and 3 to 4 ml of thionyl chloride were taken in a conical flask and maintained at a temperature of 50°C for 10-20 minutes was cooled and kept overnight. The crude product recovered was separated by filtration and was used for the preparation of various esters.

Preparation of Esters of 3-hydroxy flavones

To 0.004 moles of 3-hydroxyflavone and 0.005 moles different acid chlorides were taken in a conical flask and 4ml of dry, redistilled pyridine was added as a catalyst and maintained at a temperature of 50°C over a period of 22.5 hours, Cooled to room temperature and was added to ice cold water. The crude product separated was filtered and recrystallised from ethanol.

Antioxidant activity by DPPH Radical Scavenging Assay

Antioxidant activity of the test compounds was determined by diphenylpicrylhydrazyl (DPPH) radical scavenging method as described earlier [16]. Different aliquots of test compound (10 to 1000µg/ml) in methanol were mixed with 1 mM of methanolic DPPH solution to a final volume of 2.0 ml, incubated for 20 minutes at room temperature and absorbance measured at 517nm. Blank was carried-out in the same manner without the drug and curcumin was taken as the standard. The experiment was performed in triplicate and the percentage radical scavenging was determined as [(Blank – Test)/ Blank] x 100. EC_{50} was calculated using the Microsoft excel.

Antimicrobial Activity by Agar Diffusion Assay

Antimicrobial activity was determined by agar diffusion method explained earlier [17]. All the twelve synthesized test compounds were tested against four species of bacteria namely, *Bacillus subtilis* (gram positive) *Staphylococcus aureus* (gram positive) *Escherichia coli,* (gram negative) *Pseudomonas aeruginosa* (gram negative). The technique used was agar diffusion method using 100 µg/ 0.l ml of Amoxicillin and Gentamycin as standard. Specified quantity of beef extract, peptone and agar were accurately weighed, dissolved in distilled water and sterilised by autoc1aving at 121°C for 15 minutes. The plates were prepared with the assay media was cooled to 50°C. It was then inoculated with the test organisms. Four bores per plate were made using sterile cork borer. The above operation was carried-out under aseptic condition in sterile area.

Stock solutions of the test compounds were prepared in dimethylsulfoxide (DMSO). However, the concentration (maximum of 1%W/V) of DMSO used to dissolve the test compounds did not shown by itself any antimicrobial activity. The bores were marked accordingly. 100 µg/ 0.1 ml of the test compounds, standard and control were incubated at

37°C for 18-24 hours. At the end of 24th hour the zone of inhibition produced by the test compounds was measured using a scale. The zone of inhibition obtained by different test compounds was compared with that of standard.

Results and Discussion

Synthesis and purification

The starting material for the synthesis of 3-hydroxy floavone (NFJ-1) was O-hydroxy acetophenone. O-hydroxy acetophenone was refluxed with equi-molar proportion of p-dimethylaminobenzaldehyde in presence of ethanol and potassium hydroxide which gave chalcone. The crude chalcone obtained was purified by recrystallisation from acetone. The purity of test compound was checked by melting point determination and TLC. Further, the purified test compound was stirred with 30% hydrogen peroxide in presence of ethanol and sodium hydroxide which gave 3-hydroxy flavone (NFJ-1). The crude product was recrystallised from ethyl acetate and purity was checked by melting point determination and TLC. IR, ^{1}H-NMR and Mass spectral studies supported to establish the structure of compound NFJ-1.

Further, 3-hyroxy flavone was subjected for acetylation using acetic anhydride and methylation using dimethyl sulphate in acetone medium containing anhydrous potassium carbonate to obtain the corresponding 3-acetoxy flavone (NFJ-2) and 3'-methoxy flavone (NFJ-3) respectively. They were purified by recrystallised from ethanol. Their purity was checked by melting point determination and TLC. The structure of test compounds such as NFJ-2 and NFJ-3 was established by IR, and ^{1}H-NMR spectral studies. A series of esters of 3-hydroxy flavones were prepared by reacting with different acid chloride in presence of dry redistilled pyridine as catalyst. The crude esters recovered were purified by recrystallisation from ethanol. Further, purity of the test compounds such as NFJ-4 to NFJ-12 was checked by melting point, TLC, and IR spectral data. The yield of the final compound NFJ 1, 3-acetoxy flavone (NFJ 2) and methoxylated derivative (NFJ 3) were 58%, 70% and 68% respectively. The yield of esters of 3-hydroxy flavones was between 62 to 70% as shown in the table 1.

Table1: Compounds synthesised their molecular formula, moleculat weight, Melting point, % yield, λ_{max} and ϵ_{max}

Compound code	Molecular Formula	Mol. Wt	Melting point (°C)	% Yield	λ_{max}	ϵ_{max}
NFJ-1	$C_{17}H_{15}O_3N$	281	191	58	403	$2.80x10^4$
NFJ-2	$C_{19}H_{15}O_4N$	323	151	70	390	$2.47x10^4$
NFJ-3	$C_{18}H_{17}O_3N$	295	122	68	394	$2.65x10^4$
NFJ-4	$C_{24}H_{18}O_4NCl$	420	224	65	405	$4.38x10^4$
NFJ-5	$C_{24}H_{18}O_4NCl$	420	232	67	359	$3.14x10^4$
NFJ-6	$C_{24}H_{18}O_5N_2$	414	209	60	403.5	$2.81x10^4$
NFJ-7	$C_{24}H_{18}O_5N_2$	414	218	62	403.5	$3.87x10^4$
NFJ-8	$C_{25}H_{21}O_4N$	399	238	68	403.5	$1.84x10^4$
NFJ-9	$C_{17}H_{15}O_4N$	399	220	70	400	$3.00x10^4$
NFJ-10	$C_{24}H_{18}O_5N_2$	415	228	63	399	$2.05x10^4$
NFJ-11	$C_{24}H_{18}O_4NBr$	463	249	69	402	$2.48x10^4$
NFJ-12	$C_{24}H_{19}O_4N$	385	204	60	397.5	$2.15x10^4$

Spectral analysis

3-hydroxy-2-[((4-dimethylamino) phenyl)]-benzopyran-4-one (NFJ – 1): Yield 58%; mp 191°C; IR (KBr) ν_{max}/cm^{-1} 3240.2, 2813.9, 1600.8, 1558.4-1475.4, 1369.4, 1114.8; ^{1}H NMR (400MHz in $CDCl_3$): 7.862-7.840 [d, 2ArH(b,c)], 7.667-7.392 [m, 4ArH(d,e,f,g)], 6.9 [s, 1H, OH(h)], 6.768-6.745 [d, 2ArH(b,c)], 3.074 [s, 6H (a)]; MS m/e = 281, 253, 238, 181, 160, 141, 120, 126, 118

3-acetoxy-2-[((4-dimethylamino) phenyl)]-benzopyran-4-one (NFJ – 2): Yeild 70%; mp 151°C; IR (KBr) ν_{max}/cm^{-1} 2825, 1759, 1639.4, 1598.9-1521.7, 1365.5, 1147.6; NMR (400MHz in $CDCl_3$): 8.249- 8.230 [m, 4ArH(d,e,f,g)], 7.862-7.840 [d, 2ArH(b,c)], 7.667-7.392 [m, 4ArH(d,e,f,g)], 6.768-6.745 [d, 2ArH(b,c)], 3.074 [s, 6H (a)], 2.390 [s, 2H(h)]

3-methoxy-2-[((4-dimethylamino) phenyl)]-benzopyran-4-one (NFJ – 3): Yeild 68%; mp 122°C; IR (KBr) ν_{max}/cm^{-1} 2898.8, 1600.8, 1554.5-1146.7; NMR (400MHz in $CDCl_3$): 8.269-8.250 [m, 4ArH(d,e,f,g)], 8.128-8.105 [d, 2ArH(b,c)], 7.641-7.371 [m, 4ArH(d,e,f,g)], 6.801-6.778 [d, 2ArH(b,c)], 3.889 [s, 3H (h)], 3.086 [s, 6H(a)]

3-(4-chloro benzoyloxy)-2-[((4-dimethylamino) phenyl)]-benzopyran-4-one (NFJ – 4): Yeild 65%, mp 224°C; IR (KBr) ν_{max}/cm^{-1} 2923.9, 1764.7, 1643, 1558.4-1452.3, 1357.8, 1066.6

3-(2-chloro benzoyloxy)-2-[((4-dimethylamino) phenyl)]-benzopyran-4-one (NFJ – 5): Yeild 67%; mp 232°C; IR (KBr) ν_{max}/cm^{-1} 2923.9, 1754.7, 1648.2, 1558.4-1452.3, 1311.8, 1068.5

3-(3-nitro benzoyloxy)-2-[((4-dimethylamino) phenyl)]-benzopyran-4-one (NFJ – 6): Yeild 60%; mp 209°C; IR (KBr) ν_{max}/cm^{-1} 3082.0, 1764.7, 1647.1, 1568.0-1471.6, 1367.7, 1068.5

3-(4-nitro benzoyloxy)-2-[((4-dimethylamino) phenyl)]-benzopyran-4-one (NFJ – 7): Yeild 62%; mp 218°C; IR (KBr) ν_{max}/cm^{-1} 3081.0, 1775.7, 1647.1, 1568.0-1471.6, 1367.4, 1079.1

3-(3-methyl benzoyloxy)-2-[((4-dimethylamino) phenyl)]-benzopyran-4-one (NFJ – 8): Yeild 68%; mp 238°C; IR (KBr) ν_{max}/cm^{-1} 3047.3, 1766.7, 1641.3, 1564.2-1450.4, 1363.4, 1080.1

3-(4-methyl benzoyloxy)-2-[(4-dimethylamino) phenyl]-benzopyran-4-one (NFJ – 9): Yeild 70%; mp 220°C; IR (KBr) ν_{max}/cm^{-1} 3050.4, 1763.2, 1643.2, 1564.2-1449.2, 1363.6, 1080.1

3-(4-methoxy benzoyloxy)-2-[(4-dimethylamino) phenyl]-benzopyran-4-one (NFJ – 10): Yeild 63%; mp 228°C; IR (KBr) ν_{max}/cm^{-1} 3082.3, 1764.7, 1647.1, 1568.0-1471.4, 1143.7

3-(3-bromo benzoyloxy)-2-[(4-dimethylamino) phenyl]-benzopyran-4-one (NFJ – 11): Yeild 69%; mp 249°C, IR (KBr) ν_{max}/cm^{-1} 2864.1, 1766.7, 1643.2, 1564.2-1419.5, 1022.2, 869.8

3-(3-benzoyloxy)-2-[((4-dimethylamino) phenyl)]-benzopyran-4-one (NFJ – 12): Yeild 60%; mp 204°C; IR (KBr) ν_{max}/cm^{-1} 2825.5, 1751.1, 1639.4, 1562.2-1419.5, 1365.5, 1018.3

Antioxidant activity

The antioxidant activity was expressed in terms of EC_{50}. The EC_{50} value for the 3-hydroxy flavone was observed at 650 g/ml The EC_{50} value for the 3-acetoxy flavone and 3-methoxy flavone was observed at 1000 µg/ml and 830µg/ml respectively. The EC_{50} values for various esters of 3-hydroxy flavones were found to be in the range of 830µg/ml to 1250 µg/ml as shown in the table 2.

Table 2: Antioxidant activity: DPPH radical Scavenging assay

Compound	EC_{50} (µg/ml)
Curcumin	60
NFJ-1	650
NFJ-2	1000
NFJ-3	830
NFJ-4	1250
NFJ-5	1250
NFJ-6	835
NFJ-7	830
NFJ-8	1245
NFJ-9	1250
NFJ-10	1280
NFJ-11	840
NFJ-12	950

Antimicrobial activity

The twelve test compounds synthesised, purified and characterized were screened for their qualitative antimicrobial activity. They were tested against four species of bacteria namely *Staphylococcus aureus* (gram positive) *Escherichia coli* (gram negative) *Pseudomonas aeruginosa* (gram negative). The technique used was agar diffusion method using 100 µg / 0.l ml of Amoxicillin and Gentamycin as standard (table 3).

3-hydroxy flavonoids showed the antimicrobial activity less than that of the standard antibiotics. However, compounds such as NFJ-l and NFJ-2 showed activity greater than that of the other compounds tested. Since the antimicrobial activity of compounds such as NFJ-l and NFJ-2 were not greater or equivalent to that of the standard amoxicillin or gentamycin. It was not worthwhile to perform quantitative antimicrobial activity by 96 well plate method using ELISA reader. Hence, quantitative data has not been established for the compounds NFJ-l and NFJ-2. However, further chemical modification could prove to generate many other derivatives from the parent compound for their potential antimicrobial activity.

Table 3: Antimicrobial activity of synthesised molecules

Compound code	Zone of Inhibition (mm)			
	Bacillus subtillus	*Pseudomonus aerugenosa*	*Escherichia coli*	*Staphylococcus aureus*
Amoxycillin	27	29	34	31
Gentamycin	25	30	28	32
NFJ-1	14	12	9	11
NFJ-2	13	13	11	10
NFJ-3	10	11	10	8
NFJ-4	6	10	9	10
NFJ-5	11	11	11	9
NFJ-6	8	9	12	9
NFJ-7	7	8	10	7
NFJ-8	9	7	12	8
NFJ-9	5	6	10	8
NFJ-10	6	7	10	12
NFJ-11	5	7	11	9
NFJ-12	5	6	10	8

Conclusion

Substituted flavones were synthesised, purified, characterised and evaluated for antioxidant antimicrobial activity. The yield of all substituted flavones was found to be in the range of 60-75%. Owing to the solubility problems pKa for the test compounds were not established. They showed comparatively poorer DPPH radical scavenging activity. EC_{50} values of the test compounds were much higher than the standard curcumin. However, different radical scavenging assays would be preferred to confirm the activity. Among the compounds two of them namely, NFJ-1 and NFJ-2 showed good antibacterial activity against both gram-positive and gram-negative organism when compared to that of the standard antibiotics Amoxicillin and Gentamycin. Further, structural modification of the parent molecule may lead to better antibacterial compounds.

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