



EVALUATION OF POTENTIAL ANTIMICROBIAL ACTIVITY OF SYNTHETIC FLAVONOIDS

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Abstract

Introduction: In recent times, most of the currently available antimicrobial agents have developed resistance. Extensive pharmacological activities including bactericidal and bacteriostatic nature of flavonoids, made them as priority agents in this aspect of research study. Synthetic flavonoids such as hydroxy thiophen derivatives were considered to evaluate for antimicrobial activity in this study.

Objective: The present study involves the analysis for antimicrobial activity of thiophen substituted synthetic flavonoids.

Methods: Claisen-Schmidt method of condensation followed by oxidative cyclization reactions from substituted hydroxyacetophenone with aromatic aldehydes were used to synthesize the various analogues of flavonoid compounds. Then these compounds after their FTIR, ¹H NMR, MS spectral characterization and elemental analysis, were screened for in vitro antibacterial and antifungal activity by using disc diffusion method followed by determining their respective zone of inhibitions.

Results: All the synthesized test flavonoid compounds exhibited the good antibacterial and antifungal spectrum activity over *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* bacteria and *Candida albicans* and *Aspergillus niger* fungal microbes. However compounds such as F1, F2 and F4 showed moderately significant antibacterial activity against *P. aeruginosa* organism than the other test compounds and the same F1 and F2 test compounds exhibited significant antifungal activity at 100µg concentration.

Conclusion: The present study demonstrated that the novel thiophen substituted flavonoids (F1, F2, F3 and F4) found to have promising antimicrobial and antifungal activity which needs to be confirmed by in vivo studies.

Keywords: Antibacterial, Antifungal, Hydroxyacetophenone, Mass spectroscopy, FTIR, NMR.

Introduction

Microbial infection may be an important health issue in animals and humans in developing countries (WHO, 2020). Additionally most of the pathogenic bacteria (around 70%) are resistant to one or more of the antimicrobial drugs presently available in the market, with some bacterial strains being resistant to all antimicrobials ^[1]. As per Collier et al. 1998, it was reported that the bacterial infections in some susceptible patients, especially with HIV/AIDS can cause death. Developing countries declared that there is alarming increase of antibiotic resistance among pathogenic bacteria.

Therefore there is need for discovery of new antimicrobial drugs or leads as the number of microbes developing resistance to existing antimicrobial drugs is increasing and spreading worldwide ^[2].

The flavonoids which occur abundantly in plant kingdom as a heterocyclic organic compounds are the reliable solution to overcome this problem of bacterial resistance. Due to the multiple biological activities like antibacterial, antifungal, antiviral, anti-allergic, antiproliferative and antioxidant nature, the flavonoids are being tried to treat infectious diseases in humans ^[3]. Though natural flavonoids are highly potent, their usage in research

is reduced due to certain limitations like stability, solubility and pharmacokinetic properties. Antibacterial research is diverted towards semisynthetic and synthetic flavonoids due to their easy synthetic procedures, stable pharmacokinetics and better antimicrobial property even at lowest concentration. It's been reported that many number of flavonoids such as isoflavones, flavanones apigenin, galangin, flavone and flavonol glycosides, and chalcones were shown to possess potent antibacterial activity [3]. Since the above stated compounds seems to be novel leads, and in coming days it may allow them to be used as a new class of antimicrobial agents. A benzopyran core, with a phenyl ring connected on the C-2, C-3 or C-4 atoms is the vital chemical shape found in most of the flavonoids [5]. However, the biological activities of synthetic analogues of gamma benzopyranes with smaller heterocyclic substitutions such as thiophen, furan etc., in place of phenyl group at 2- position have not been fully exploited. R.S. Keri et al. 2017 reported that, replacement of phenyl ring by thiophen moiety resulted in the increase of biological activity. [6].

Hence the above said literatures directed us to explore the biological activities associated with the modified benzopyran nucleus [7]. With this background the study of gamma benzopyran with various substitutions on the ring A and B such as 3 hydroxy thiophene flavones which were synthesized to explore for their antibacterial and antifungal activities was considered. The antibacterial and

antifungal activity of the test compounds was aimed to determine the zone of inhibition values of various pathogenic bacteria.

1. Material and Methods

1.1 Materials: The chemical substances used were of laboratory reagent (LR) grade and analytical reagent (AR) purchased from Loba Chemicals, Qualigens, NR Chemicals, Lancaster, Sigma, Merck and Hi-Media Reachem, SD Fine Chemicals Ltd. Substituted hydroxyacetophenone and benzaldehyde were purchased from Sigma Aldrich, USA Chemical Company. TLC plates were from Merck 60 F254, Darmstadt Germany. Solvents and chemicals like ethanol, n-hexane, ethyl acetate, DMSO of extra pure analytical grade were purchased from S.D. Fine Chemicals. Mueller-Hinton agar and Dextrose Agar media were purchased from Hi-media, USA. Ciprofloxacin and Fluconazole were procured from pharmaceutical industries.

1.2 Synthesis of test flavonoid compounds.

The flavonoids containing benzopyran-4-one were synthesized by two steps. In first step, an intermediate chalcone was prepared by condensing respective acetophenone with a substituted aldehyde in basic medium according to a Claisen-Schmidt [8] condensation. Next synthesized chalcone was exposed to oxidative cyclization reaction with hydrogen peroxide as per method of Algar-Flynn-Oyamada [9-13].

Table 1: Different substitution pattern on ring A and B of the synthesized compounds

Sl.No	Code	R	R ¹	R ²	R ³	R ⁴
1	F1	H	OCH ₃	H	H	H
2	F2	H	OCH ₃	H	CH ₃	H
3	F3	CH ₃	OCH ₃	H	H	H
4	F4	CH ₃	OC ₂ H ₅	H	CH ₃	H

2.3 Analytical methods:

Melting points were determined by using open capillaries in degree Celsius and uncorrected. FT-IR spectrometer-8300 (Shimadzu, Japan) and FTIR-4100 were used to record IR spectra under KBr disc method. ¹H NMR spectra of the test compounds were recorded in CDCl₃ or DMSO solution at 400 MHz on AMX-400 MHz High Resolution Multinuclear FT-NMR Spectrometer (Bruker) with tetramethylsilane (TMS) as internal standard. Mass spectra of the test compounds were recorded on GC-MS-QP5050A (Shimadzu) at quality assurance

department, Manipal College of Pharmaceutical Sciences (MCOPS). The compounds were analyzed for elemental analysis. Physical data and the spectral data of the compounds are recorded in Table-2.

2.4 Antibacterial activity: [14-16]

The antibacterial and antifungal screening of the 2 thiophen flavonoid derivatives were performed through the agar well diffusion method against *Bacillus subtilis*, *Staphylococcus aureus* (Gram-positive bacteria), *Escherichia coli*, *Pseudomonas*

aeruginosa (Gram-negative bacteria) and fungi strains like *Aspergillus niger* and *Candida albicans*. These organisms were obtained from the Department of Pharmaceutical Biotechnology, Manipal College of Pharmaceutical School (MCOPS) for the study. The Petri dishes were kept at 160-170°C till 30 min before use for sterilization. For bacteria approximately 20 ml of sterile M-H Agar and for fungus 20ml of Sabouraud Dextrose Agar (SDA) medium were poured in sterile Petri plates up to a depth of 4-5 mm and allowed to solidify. The sterile cotton swabs after dipping into the bacterial culture and fungal culture (106 to 108 CFU/ml) respectively. The inoculation was done by swabbing evenly over the above said agar plates, by using a sterile cork-borer (6 mm diameter). Three cylindrical cavities were made within the medium, one for synthesized compounds, second cavity for positive control, i.e., standard drug (Ciprofloxacin for bacteria and Flucanazole for fungi) and third for negative control DMSO. Care was taken for the temperature of agar and SDA medium not to exceed 48-50°C during study. Inoculation, temperature of the medium in the dishes was kept at a 37°C. The varied concentrations of flavone derivatives and standard drug sample solutions in DMSO were

incorporated within the cavity or holes. A constant amount 0.1 ml (100 µg/ml) of the test and standard solution were added to cavity of the cup and allowed them for 30 min refrigeration to diffuse. The plates were incubated at 37°C for 24hour to bacteria; 28°C for 48hour (fungi). The typical zone of inhibition was recorded by excluding the well, after preparing the triplicate plates for each. A negative control DMSO was used. The range of zone of inhibition in millimeters (mm) of the test flavonoid compounds was compared with the standard drugs to assess the antibacterial potential.

2. Results and Discussion:

2.1 Chemistry:

Condensation followed by oxidative cyclization reaction was used to synthesize the flavonoids according to method of Algar-Flynn-Oyamada reaction^[17-19]. 2-Hydroxy acetophenone in methanol under aqueous alkali media, was refluxed with different substituted benzaldehyde (aldol condensation), to obtain 70–80% of corresponding chalcones yield. The chalcones in methanol base after cyclization reaction with hydrogen peroxide gave the corresponding flavones.

Table 2: Experimental data of thiophen derived synthetic flavonoids

Code	Molecular formula	MW	% yield	Melting point (°C)	R _f Value	Elemental analysis			
						C	H	O	S
F1	C ₁₄ H ₁₀ O ₄ S	274	45	223-226	0.50	63.92%	3.30%	19.65%	13.13%
F2	C ₁₅ H ₁₂ O ₄ S	288	45	228-232	0.55	62.49%	4.20%	22.20%	11.12%
F3	C ₁₅ H ₁₂ O ₄ S	288	45	218-222	0.43	62.49%	4.20%	22.20%	11.12%
F4	C ₁₇ H ₁₆ O ₄ S	316	50	234-238	0.66	4.54%	5.10%	20.23%	10.14%

* n-hexane / ethyl acetate (9.5:0.5)

2.1 Spectral characterization (IR, ¹H NMR and Mass spectral data) of thiophen derived synthetic flavonoids:

- 1) F1. 3-hydroxy-7-ethoxy-2-thiophen-2-yl-4H-chromen-4-one

IR (KBr) 3267 (b, OH), 2922, 1610 (C=O), 1452, 1253 and 1116 cm⁻¹.

¹H NMR (400 MHz, CDCl₃) δ 3.94 (3H, s, H-OCH₃), 6.50 (1H, m, H-Ar), 6.99– 8.13 (5H, m, H-Ar) and 13.43 (1H, s, H-OH).

Mass (E I) m/z 275 (M+1)+

- 2) F2. 3-hydroxy-7-methoxy-2-(3-methylthiophen-2-yl)-4H-chromen-4-one

IR (KBr) 3217 (b, OH), 3064, 1600 (C=O), 1452, 1257 and 1168 cm⁻¹.

Mass (E I) m/z 289 (M-1) +

- 3) F3. 3-hydroxy-7-methoxy-8-methyl-2-thiophen-2-yl-4H-chromen-4-one

IR (KBr) 3267 (b, OH), 3190, 1610 (C=O), 1564, 1452, 1271 and 1168 cm⁻¹.

$^1\text{H NMR}$ (400 MHz, DMSO) δ 2.33 (3H, s, H-CH₃), 3.94 (3H, s, H-OCH₃), 6.64 – 8.03 (5H, m, H-Ar) and 9.98 (1H, br s, H-OH).

Mass (C I) m/z 288 (M)+

- 4) F4. 7-ethoxy-3-hydroxy-8- methyl-2-(3-methyl thiophen- 2-yl)-4H-chromen-4-one

IR (KBr) 3342 (b, OH), 2924, 1602 (C=O), 1448, 1274, 1217 and 1134 cm^{-1} .

$^1\text{H NMR}$ (400 MHz, CDCl₃) δ 1.55 (3H, m, H-CH₃), 2.38 (3H, s, H-CH₃), 2.56 (3H, s, H-CH₃), 4.21 (2H, q, H-CH₂), 6.99 (1H, d, $J = 5.2$ Hz, H-5'), 7.02 (1H, d, $J = 8.8$ Hz, H-6), 7.49 (1H, d, $J = 5.2$ Hz, H-4') and 8.09 (1H, d, $J = 8.8$ Hz, H-5).

Mass (E I) m/z 317(M+1)+

2.2 Spectral study:

The IR spectrum of synthesized test compound showed prominent optical phenomenon suggesting the presence of carbonyl group (C=O). The molecular ion peaks obtained within the mass spectra of those synthesized benzopyranes were according to the relative molecular mass of the respective compounds.

2.3. Antimicrobial Activity:

Nowadays the number of studies concerning evaluation of antimicrobial properties of plant extracts rich in phenolic compounds, including flavonoids and phenolic acids has been increased. However, the antimicrobial activities of such individual pure compounds are relatively less due to enormous wealth of species and natural substances [20]. In literature study, only a few flavonoid

glycosides such as vitexin, isovitexin, vitexin 2''-O-rhamnoside, orientin, and isoorientin have been studied for antimicrobial activity [21, 22]. In this context there is still a need for analysis of synthetic flavones against the some bacterial and fungal strains by a standardized method. In this study, substituted synthetic flavones based on their zone of inhibition, exhibited the moderate to good antimicrobial activity (Bacteria and Fungi).

The synthesized flavone compounds antibacterial and antifungal activity results, in respect to their zone of inhibition are tabulated in Table 3 and 4. It is reported that the antibacterial response may increase or decrease based on substitution in the ring A and ring B. Results from the study reveal that addition of hydroxyl at ring A, methyl at ring B and thiophen at C ring enhances the antibacterial response against Gram positive and Gram negative bacteria anti antifungal activities [22].

2.3: 1. Antibacterial activity (zone of inhibition) of thiophen derived synthetic flavonoids against *B. subtilis*:

All the 4 synthesized test compounds [2-thiophen-2-yl-3-hydroxy- γ -benzopyranes] evaluated for antibacterial activity against *B. subtilis* at 100 $\mu\text{g/ml}$ concentration, were found significantly active, exhibiting their zone of inhibition in the range of 15 – 18 mm as shown in table -3. Test compounds such as F1 and F3, showed their zone of inhibition at 18mm, where as F2 zone of inhibition was 16 mm when compared to the standard drug ciprofloxacin and control DMSO as shown in table -3. The control drug DMSO showed nil/no zone of inhibition as contrast to the standard drug ciprofloxacin which showed 32mm.

Table 3: Antibacterial and Antifungal activity (zone of inhibition in mm) of thiophen derived synthetic flavonoids

S. No	Code	Zone of inhibition (mm) at 100 μg concentration against bacterial strains				Zone of inhibition (mm) at 100 μg concentration against Fungal strains	
		<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>Candida albicans</i>	<i>Aspergillus niger</i>
1	F1	18	18	17	16	16	18
2	F2	16	18	14	15	13	18
3	F3	18	18	18	NA	10	NA
4	F4	18	16	16	NA	NA	NA
5	Ciprofloxacin	32	36	30	27	18	25
6	DMSO	-	-	-	-	NA	NA

NA-Not active, - No zone of inhibition of microbial growth, DMSO-Dimethylsulfoxide.

2.3:2. Antimicrobial activity (zone of inhibition) thiophen derived synthetic flavonoids against *S. Aureus*:

Out of 4 test compounds [2-thiophen-2-yl-3-hydroxy- γ -benzopyranes] evaluated for antibacterial activity against *S. aureus* at 100 μ g/ml concentration, except F4 all the test flavonoid compounds such as F1, F2 and F3 showed significant activity having the zone of inhibition in the range of 18 mm as in the table-3, suggesting that the test flavonoid compounds exhibit good antibacterial activity against *S. aureus*.

2.3: 3. Antibacterial activity (zone of inhibition) of thiophen derived synthetic flavonoids against *E. coli*:

Out of the 4 test compounds [2-thiophen-2-yl-3-hydroxy- γ -benzopyranes] evaluated for antibacterial activity against *E. coli* at 100 μ g/ml concentration, their zone of inhibition was within the the range of 14 -18mm which is lesser than that of standard drug ciprofloxacin (30mm). Out of 4 synthesized test compounds only F3 compounds showed the zone of inhibition of 18 mm, which is significant among other compounds as shown in *table-3*. Test compounds such as F1 and F4 exhibited zone of inhibition at 17 mm and 16 mm respectively. Except F2 other test compounds such as F1, F3 and F4 showed good antibacterial spectrum activity against *E. coli*.

2.3: 4. Antibacterial activity (zone of inhibition) of thiophen derived synthetic flavonoids against *P. Aeruginosa*:

Out of 4 test compounds [2-thiophen-2-yl-3-hydroxy- γ -benzopyranes] evaluated for antibacterial activity against *P. aeruginosa*, test compounds such as F1, F2 exhibited the zone of inhibition in the range of 14-16mm as compared to the standard drug ciprofloxacin (27 mm) shown in the *table-3*. Whereas test compounds such as F3 and F4 showed nil zone of inhibition at 100 μ g/ml concentration as compared to control DMSO shown in *table-3*. However, compound such as F1 showed significant zone of inhibition (16 mm) as shown in the *table 3*.

2.4. Antifungal activity thiophen derived synthetic flavonoids:

All the synthesized thiophen derived flavonoids were screened for their antifungal activity by agar diffusion method using fluconazole as standard antifungal agent. The antifungal activity was tested at 100 μ g/ml concentration against *Candida albicans*

and *Aspergillus niger*. The diameter of zone of inhibition was measured and rounded off to the closest integer (mm) and are shown in table 3. The test compounds which showed zone of inhibition over and above 14 mm were considered as significantly active.

2.4: 1. Antifungal activity of thiophen derived synthetic flavonoids against *Candida albicans*:

Out of 4 synthesized test compounds [2-thiophen-2-yl-3-hydroxy- γ - benzopyranes] evaluated for antifungal activity against *Candida albicans*, 3 compounds such as F1, F2 and F3 showed significantly active zone of inhibition in the range of 10-16 mm as seen in the table 3. Test compound F1, 2-thiophen-2-yl-3-hydroxy- γ -benzopyrone showed maximum zone of inhibition up to 16 mm as compared to other test compounds when compared to standard drug fluconazole 18mm. Whereas F4 compounds zone of inhibition was nil, suggesting that the compound has no antifungal activity against *Candidia albicans* as shown in the *table 3*.

2.4: 2. Antifungal activity of thiophen derived synthetic flavonoids against *Aspergillus niger*:

Out of 4 synthesized test compounds [2-thiophen-2-yl-3-hydroxy- γ - benzopyranes] evaluated for antifungal activity against *Aspergillus niger*, only 2 compounds such as F1 and F2 showed the maximum zone of inhibition in the range of 18 mm which was shown in the *table-4*. Test compounds such as F3 and F4 showed nil/no zone of inhibition compared to that of standard drug fluconazol and control DMSO as given in the *table- 4*.

3. Conclusion:

Resistance to antimicrobial agents has become a major problem globally and needs immediate attention. The general trend is to modify structurally the existing classes of drugs to which resistance has developed. Even this method has not been very fruitful and hence the focus is now on development of new classes of drugs that work at different targets [23].

The antibacterial activity of the synthesized compounds in this study was considered with respect to their zone of inhibition at their growth, and also considered that the compounds showing zone of inhibition more than 14 mm were more significant. Because it is reported that the antibacterial ability was classified according to the diameter of zone of inhibition (DIZ) as follows: not

sensitive (DIZ < 8.0 mm), moderately sensitive (8.0 < DIZ < 14.0 mm), sensitive (14.0 < DIZ < 20.0 mm), and extremely sensitive (DIZ > 20.0 mm)^[16, 24]. According to this the synthesized flavonoid compounds such as F1 is efficacious in antifungal activity against *Candida albicans* and *Aspergillus niger* as compared to other synthesized flavones in the study. Whereas F1 and F2 synthesized compounds have been shown sensitive antifungal activity against *Aspergillus niger* while standard fluconazole was shown extremely sensitive activity.

Similarly, in, the synthesized flavonoid compounds such as F1, F2, F3 and F4 have shown more significant antibacterial activity against the gram positive and gram negative bacteria such as *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* but lesser than the standard drug ciprofloxacin. However test compounds such as F2 and F4 had less/no zone of inhibition antibacterial activity against *E. coli* and *P. aeruginosa* as compared to other compounds and F3 and F4 have showed nil zone of inhibition against the *P. aeruginosa* gram negative organism.

In the present study, the synthesis of four 2-thiophen analogues of synthetic flavonoids was done, for characterization study to establish the structure. Most of the compounds have shown good to moderate antimicrobial activity, while some of the thiophen analogue flavones were found to elicit significant antibacterial activity against Gram positive and Gram negative bacteria and antifungal activity against two strains of fungi. The antimicrobial activity exhibited by these thiophen derived synthetic flavonoids, may be due to the substitution of dimethyl, methoxy or ethoxy groups in addition to the hydroxyl group present at 3-position in each member. It was clear from the data that the presence of hydroxyl group at 3-position in ring C, greatly influences antibacterial and antifungal activity[12].

The study revealed that 3-hydroxy thiophen flavone derivatives were found to be more active against Gram positive bacteria, while the synthetic flavanone derivatives such as F1, F2 and F3 showed the good spectrum activity against *E. coli* organisms. But for *P. aeruginosa* Gram negative organisms the compounds F3 and F4 did not show any antibacterial zone of inhibition. However, further study is needed for the in-vivo antimicrobial activity and toxicity studies. The results obtained from this study can be used as research source for further development of new antimicrobial agents.

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