# STUDIES ON *ENTEROCOCCUS FAECIUM* GROWTH - INHIBITORY ACTION OF 1,5-BIS (2-HYDROXYPHENYL)PENT-1,4-DIENE-3-ONE AND RELATED COMPOUNDS: A SEARCH FOR ENVIRONMENTALLY BENIGN ANTI-BACTERIAL AGENT

## Archna Rani[*] and Sapna Jain
Department of Applied Chemistry, Delhi College of Engg.,
University of Delhi, Delhi-110042, India.
E-mail: archna.rani@gmail.com

## ABSTRACT

*Following the basic concept of Green chemistry the present work is aimed at synthesis of compounds and evaluating their antibacterial action against clinically isolated resistant pathogenic strain of Enterococcus faecium.Thus a conjugated carbonyl compound, (**1a**; 1, 5-bis(2-hydroxyphenyl)pent-1,4-diene-3-one) was synthesized as a representative compound by incorporating reported basic bioactive chemical moieties and various transformations in the structure has also been made, as, absence of hydroxyl group (**2a**;1,5-Diphenylpent-1,4-diene-3-one), extension of conjugation (**3a**;1,9-Diphenylnon-1,3,6,8-tetraene-5-one), introduction of dimethyl amino group (**4a**;1,5-bis[4-(N,Ndimethyl)phenyl] pent-1,4-diene-3-one), replacement of benzene ring with heteroaromatic ring (**5a**;1,5-difuranpent-1,4-diene-3-one). All the compounds were screened for the first time against E.faecium. In preliminary test **1a** exhibited encouraging result, it was therefore further studied for bacteriostatic action by turbidimetric kinetic methods. Growth kinetic curves of E.faecium were made in nutritive broth added with increasing concentration of the compound .Its Minimum Inhibitory Concentration (65µg ml$^{-1}$) was evaluated graphically. The activity of **1a** was found to correlate with its structural resemblance with chalcones, presence of conjugated carbonyl group and presence of hydroxyl group at 2 and 2'on the benzene ring. In the absence of hydroxyl group, only increase in number of double bonds (**3a**) is not essential in eliciting the activity but associated with increasing it. Compound **1a** may be of great interest due to its bacteriostatic property as well as its synthesis by straight forward reaction at room temperature.*

**Keywords:** *Conjugated carbonyl compounds, chalcones, E.faecium, bacteriostatic action, antibacterial agent.*

## INTRODUCTION

In the present time, a wide variety of chemicals are being used to control infectious diseases. But their hazardous nature, use of harmful chemicals as catalysts and solvents for their synthesis, are of prime concern for environmentalists. Also development of rapid resistance in pathogenic microbes is emerging out as a serious problem before the biologists.Thus designing of environmentally benign potential antimicrobial agent is a pressing need of today and is still a challenge before the scientists, worldwide.

As a result the compounds of natural origin and their derivatives are getting intense interest of the researchers worldwide for development of new class of eco-friendly antimicrobial agents. Flavanoids, in this regard, have attracted considerable attention as they possess a wide range of biological activities[1-3], such as antimicrobial[4-7], antiviral[8,9], anti-inflammatory [10,11], bactericidal and bacteriostatic action [12] and other therapeutic applications [13].

Chalcones, a type of flavanoids, in particular, are studied exhaustively by many scientists[14-16] for their antimicrobial effect. Also in various independent investigations it has been observed that cinnamic acid and its derivatives[17] and compounds having carbonyl group in conjugation with double bonds exhibit various biological activities[18]. Keeping all these points in view an attempt has been made in the present work to design a compound incorporating the above mentioned parameters in the basic structure of chalcone moiety (A). Thus 1, 5-bis (2-hydroxyphenyl)pent-1,4-diene-3-one), (**1a**), was synthesized as a

representative compound   and screened against *E.faecium.* To know the effect of varying the molecular structure of **1a** on its bioactivity, compounds **2a** (1,5-Diphenylpent-1,4-diene-3-one), **3a** (1,9-Diphenylnon-1,3,6,8-tetraene-5-one) , **4a**(1,5-Bis[4-(N,Ndimethyl)phenyl] pent-1,4-diene-3-one) and **5a** (1,5-difuran pent-1,4-diene-3-one) were synthesized and  screened against test microbe.
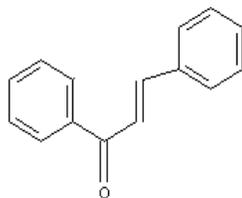


Chalcone (A)

**EXP[ERIMENTAL**

*Synthesis of compounds* (summarized in scheme1)**:**
All the reagents used for synthesis were of high purity.
 The synthesis of the chemical compounds was carried out as outlined in Scheme 1. To complete the reaction shown in Scheme 1, 0.01 mole of aldehyde was dissolved in 10 ml of ethanol and 10 ml 3M NaOH. To this reaction mixture, 0.02 moles of acetone was added slowly in an incubator-shaker at room temperature. After half an hour, the reacting components become a pale yellow solution, which in turn converted cloudy after about 10 minutes. The precipitate was filtered out and re-crystallized by appropriate solvent [19].

$$R\text{-}CHO + CH_3COCH_3 \xrightarrow{\text{NaOH}} R\text{-}CH=CH\text{-}CO\text{-}CH=CH\text{-}R$$
$$(1a-5a)$$
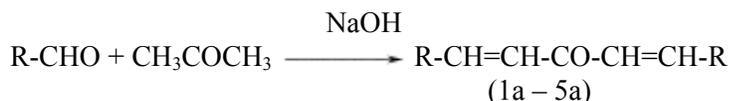
**Scheme-1**

Where R is one of the following groups-

| | |
|---|---|
|  | 1,5–bis(2–hydroxyphenyl)pent–1,4–diene–3–one (1a) |
|  | 1,5-Diphenyl pent-1,4-diene-3-one  (2a) |
|  | 1,9-Diphenylnon-1,3,6,8-tetraene-5-one (3a) |
|  | 1,5-difuran pent-1,4-diene-3-one (4a) |
|  | 1,5–di[4–bis(N,Ndimethyl)phenyl]pent–1,4–diene–3–one (5a) |

### Physical and Spectr data of synthetic compounds

The melting points for all the compounds were determined in open capillary tube. Thus they are reported as uncorrected. The infra red (I. R.) spectra were of all the compounds were recorded on Perkin Elmer BX-II Spectrophotometer (Perkin Elmer, Boston MA) using potassium bromide (KBr) pellets. The ultra violet (U.V.) Spectra were recorded on a UNICAM UV-4 Spectrophotometer using ethanol as solvent (Table 1)

**Table-1**: Physical data and their characterization of compounds.

| COMPOUND | MOLECULAR FORMULA CHN analysis %obs. (% calc.) | MELTING POINT | YEILD | IR SPECTRA | UV SPECTRA (cm$^{-1}$) |
|---|---|---|---|---|---|
| **1a** | $C_{17}H_{14}O_3$ 75.39, 5.1 (76.69, 5) $\varepsilon_{max} = 1.8$ | 112 $^o$C | 67 % | 1616.30 1641.54 3064.90 | $\lambda_{max} = 327.0$ nm $\varepsilon_{max} = 1.3$ $\lambda_{max} = 282.5$ nm |
| **2a** | $C_{17}H_{14}O$ 86.6, 5.2 (87.17, 5.9) | 110$^o$C | 88% | 1601.46 1658.21 3025.65 | $\lambda_{max} = 300.5$ nm $\varepsilon_{max} = 2.2$ |
| **3a** | $C_{21}H_{18}O$ 89.01, 5.2 (88.11,5.5) $\varepsilon_{max} = 0.8$ | 96 $^o$C | 78% | 1585.02 1653.76 3025.15 | $\lambda_{max} = 338.0$ nm $\varepsilon_{max} = 0.6$ $\lambda_{max} = 255.0$ nm |
| **4a** | $C_{21}H_{26}ON_2$ 77.9,7.8,8.9 (78.2, 8, 8.6) | 130 $^o$C | 90 % | 1578.82 1674.37 2906.07 | $\lambda_{max}$ 300.5 nm $\varepsilon_{max} = 2.3$ |
| **5a** | $C_{13}H_{10}O_3$ 72, 4.3 (72.89, 4.6) | 118 $^o$C | 75 % | 1606.94 1682.94 3119.52 | $\lambda_{max} = 312.0$ nm $\varepsilon_{max} = 2.2$ |

### Microbial assay: *Filter Paper Disc Method (Preliminary test)*
 ### Microbial strain

The Nutrient broth and Agar used were of following specifications HI MEDIA M 002 and HI MEDIA, RM 026 respectively.

*E.faecium* was procured from All India Institute of Medical Sciences (AIIMS), Delhi, India and maintained in laboratory by successive subcultures in Nutrient agar media at 4°C and by liofilazation.

### Preparation of test compound: The compounds were dissolved in DMSO.

### Preparation of plates and microbial assay:

A loop full of the given test strain was inoculated in 25ml of NB and was incubated for 18h in an incubator shaker (INNOVA 4300) at 37°C in order to obtain an active colony of bacterial strain. The plates were prepared by dissolving 13 gm of NB and 20 g of Agar in 1000ml of distilled water. In order to proceed 28-30 ml of autoclaved NB Agar media was added into (100mm) diameter Petri plates, inoculation of test strain was done by spreading(100μl per plate).Then Whatman filter paper (No.1) sterile discs  previously soaked in a known concentration of test compounds (1a-5a) were placed in nutrient agar media. The antibacterial activities were determined by the inhibition zone formed by these compounds.

*Turbidimetric kinetic method:*
After the preliminary identification of activity of compounds the turbidimetric method was employed to identify the bacteriostatic or bactericidal activity of the active compound **1a**.
An 18h culture of *E.faecium* in slant agar were transferred to 25 ml of Nutrient Broth and incubated for 18 h at 37°C with permanent stirring in order to be used as inoculums.
Erlenmeyer containing 100ml of culture medium with increasing drug concentration, to be tested, were inoculated with 400 µl of inoculum and stirred in the incubator shaker at $37^0$C, including one with solvent (DMSO) only.
Aliquots were extracted at regular intervals during 6 hours and optical density was registered in UV-VISIBLE spectrophotometer (UV Pharmaspec-1700, UV –VIS
Spectrophotometer, Shimactzu).

## RESULTS And DISCUSSION

*Preliminary screening*
The results (table 2) established compound **1a** as potent *E.faecium* growth inhibiting agent. Any manipulation in molecular structure lessens the bioactivity (**3a**) or make the compounds completely inactive (**2a, 4a, 5a**).

**Table-2**: The inhibition zones created by compounds

| COMPOUND | INHIBITION ZONE |
|----------|-----------------|
| A | - |
| 1a | 10 mm |
| 2a | - |
| 3a | 7 mm |
| 4a | - |
| 5a | - |

*Turbidimetric kinetic method*
According to well established microbial growth law,
$$\ln N_t = \ln N_0 + \mu t \quad \text{.......1}$$
where t: time in minutes, $N_t$; cfu ml$^{-1}$ at time t, $N_0$ : cfu ml$^{-1}$ at time t = 0, $\mu$ = specific growth rates in min$^{-1}$.It is reported that optical density (OD) of bacterial colony is proportional to the number of cells present. An increase in OD corresponds to cell growth and equation (2) can be used to monitor changes in viable cell count with time as function of optical density[20].
$$\ln OD_t = \ln OD_0 + \mu t \quad \text{........2}$$
The Representative compound (**1a**) has been studied for its effect on the growth of *E.faecium* at different concentration considering equation (2). The reults are depicted in Table 3.
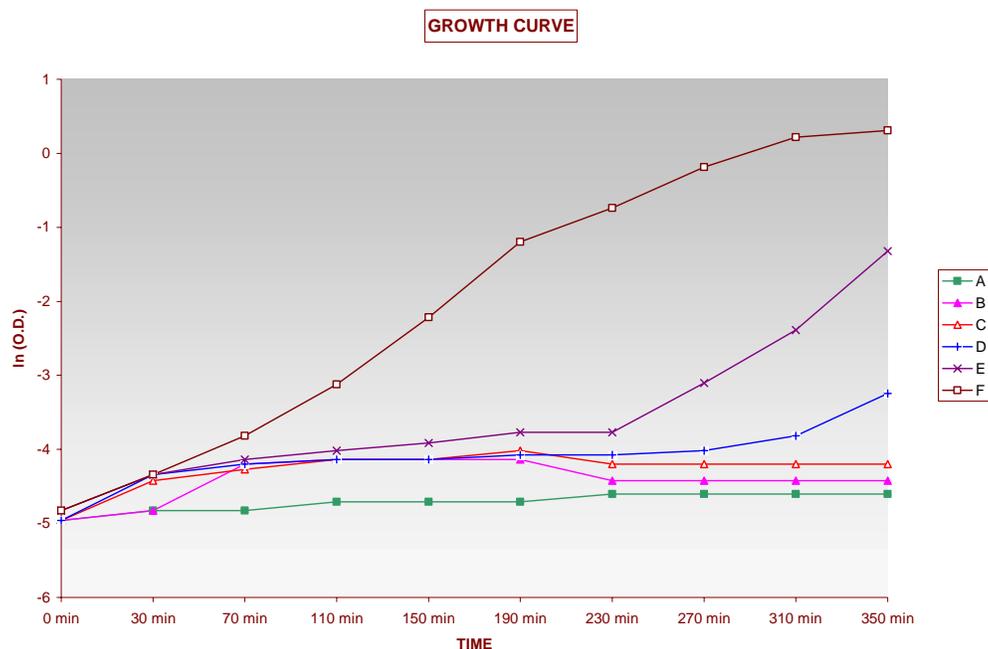Graphical representation of growth of *E.faecium* has been shown in fig. (1).

**Table-3:** Growth of *E.faecium* in presence of  **1a** (1,5-Bis(2-hydroxyphenyl)pent-1,4-diene-3-one).

| Concentration ($\mu$g ml$^{-1}$ of NB) | 70 | | 60 | | 50 | | 40 | | 30 | | 0 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EXTRACTION TIME | OD | ln(OD) | OD | ln(OD) | OD | ln(OD) | OD | ln(OD) | OD | ln(OD) | OD | ln(OD) |
| 0min | 0.007 | -4.9618 | 0.007 | -4.9618 | 0.007 | -4.9618 | 0.007 | -4.9618 | 0.008 | -4.8283 | 0.008 | -4.8283 |
| 30min | 0.008 | -4.8283 | 0.008 | -4.8283 | 0.012 | -4.4228 | 0.013 | -4.3428 | 0.013 | -4.3428 | 0.013 | -4.3428 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 70min | 0.008 | -4.8283 | 0.015 | -4.1997 | 0.014 | -4.2686 | 0.015 | -4.1997 | 0.016 | -4.1351 0.022 | -3.8167 |
| 110min | 0.009 | -4.7105 | 0.016 | -4.1351 | 0.016 | -4.1351 | 0.016 | -4.1351 | 0.018 | -4.0173 0.044 | -3.1235 |
| 150min | 0.009 | -4.7105 | 0.016 | -4.1351 | 0.016 | -4.1351 | 0.016 | -4.1351 | 0.020 | -3.912  0.109 | -2.2164 |
| 190min | 0.009 | -4.7105 | 0.016 | -4.1351 | 0.018 | -4.0173 | 0.017 | -4.0745 | 0.023 | -3.7722 0.302 | -1.1973 |
| 230min | 0.010 | -4.6051 | 0.012 | -4.4228 | 0.015 | -4.1997 | 0.017 | -4.0745 | 0.027 | -3.7722 0.478 | -0.7381 |
| 270min | 0.010 | -4.6051 | 0.012 | -4.4228 | 0.015 | -4.1997 | 0.018 | -4.0173 | 0.045 | -3.101  0.831 | -0.1851 |
| 310min | 0.010 | -4.6051 | 0.012 | -4.4228 | 0.015 | -4.1997 | 0.022 | -3.8167 | 0.092 | -2.3859 1.245 | 0.2191 |
| 350min | 0.010 | -4.6051 | 0.012 | -4.4228 | 0.015 | -4.1997 | 0.039 | -3.2441 | 0.267 | -1.3200 1.360 | 0.3071 |

OD is optical density



**Figure-1**: Growth of *E.faecium* in media containing 1,5-Bis(2-hydroxyphenyl) pent-1,4-diene-3-one, at the indicated concentration (■)70 μg ml$^{-1}$ , (▲) 60μg ml$^{-1}$,( △) 50μg ml$^{-1}$, (+) 40 μg ml$^{-1}$ (X) 30μg ml$^{-1}$, (□)0μg ml$^{-1}$.

The structure of representative compound **1a** was designed by incorporating a double bond in conjugation with carbonyl group and intoduction of two hydroxyl groups in the basic structure of chalcone (A) itself which is inactive against the test microbe. Any modification in its basic structure greatly affects its biological activity (shown in table 2) as bacterial inhibition action is completely diminishes in absence of hydroxyl group at 2 & 2' position of benzene ring (**2a**).Similar results regarding the relationship between biological activity and presence of hydoxyl groups were also noted earlier by scientists[21] in their studies. Further in the system of **2a** the incorporation of extended conjugation (**3a**) has a little effect in enhancing the bioactivity. These results indicate clearly that only incorporation of double bond is not responsible for enhanced bioactivity of the compounds but the presence of two hydroxyl groups in the ring with extended double bond is the key factor for the significant activity of compound, as in **1a**. Varying the substituent (**4a**) and replacement of benzene ring with furan (**5a**) causes complete inactivity of the compound.

The present work has been extended to study the growth of *E.faecium* at various concentration of representative compound **1a** for 6hs. It has been observed that the growth of bacteria decreases tremendously in the presence of compound **1a** in culture broth as compared to the culture without it (fig 1).At concentration 30 μg ml$^{-1}$ compound **1a** inhibits the growth of experimental bacteria marvelously (fig1, growth curve E). It is also observed that upto 230 minutes of its application ,at the same

concentration, the grwoth of the test microgbe remians almost static.Thus 230 minutes can be considered as Maximum Inhibitory Period (MIP) for the compound at this particular concentration.  At higher concentration (40 µg ml^{-1}) the same pattern has been found, with MIP about 310 minutes (Growth curve D).

From concentration 50 µg ml^{-1} to70 µg ml^{-1} no sharp rise in the curve, upto six hours of experimental time, explains the bacteriostatic property of the compound.(growth curves C,B,A resp. )

The results can further be visualized with the help of specific growth rate of  *E.faecium* with increasing concentration of compound **1a** in media. The values were obtained from ln OD vs t plots in exponential growth phase, following the equation (3) as reported earlier[22].

$$\mu = \mu_T - k \cdot C \qquad ........(3)$$

Where $\mu$: specific growth rate (min-1);    $\mu_T$: specific growth rate in medium without drug (min^{-1}) (control); k: specific inhibition rate (ml µg^{-1}.min^{-1}) and C: drug concentration (µg ml^{-1}).

The specific growth rate as function of concentration of compound 1a has been represented graphically in (fig 2) (table 4).

**Table-4:** Specific growth rate as function of concentration of the drug

| CONCENTRATION (µg ml^{-1}). | 70 | 60 | 50 | 40 | 30 | 0 |
|---|---|---|---|---|---|---|
| SPECIFIC GROWTH RATE (x10^{3}) | 0.00 | 1.60 | 3.60 | 6.40 | 15.32 | 21.80 |
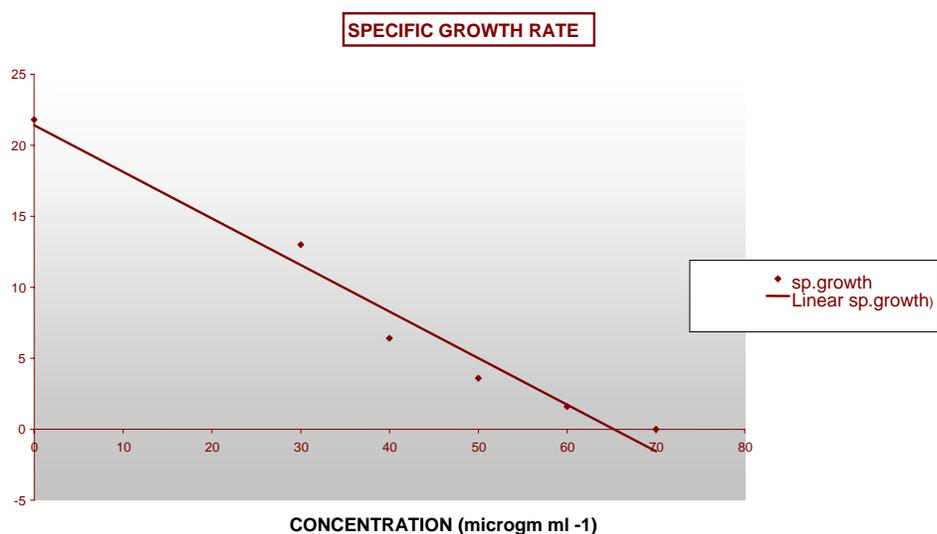


**Figure- 2:** Graphical representation of determination of MIC (minimal inhibitory concentration) by extrapolating at abscissa when µ= 0

Minimal inhibitory concentration (MIC) evaluated by extrapolating at μ=0. The MIC values obtained for the compound tested in our study was 65 μg ml$^{-1}$. Compound **1a** may be of great interest for further studies due to its bacteriostatic property rather then bactericidal effect.

We may conclude that the most important aspect of our current study lies in the designing of basic structure of the compounds which can effectively be used as bacterial growth inhibitors at very low concentration, and also can be synthesized by straightforward reaction, at room temperature avoiding the use of hazardous chemicals as solvent or catalyst or formation of any side product. It is one of the basic principles of "Green Chemistry".

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