

# Analgesic, Antibacterial and Antiviral Activities of 2-(5-Alkyl-1,3,4-oxadiazol-2-yl)-3H-benzo[f]chromen-3-ones

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Raghavendra, *et al.*: Bioactive properties of 2-(5-alkyl-1,3,4-oxadiazol-2-yl)-3H-benzo[f]chromen-3-ones

A novel series of 2-(5-alkyl-1,3,4-oxadiazol-2-yl)-3H-benzo[f]chromen-3-ones (4a-e) have been evaluated for analgesic, antibacterial and antiviral activities. Analgesic activity was carried out using acetic acid-induced writhing method in Swiss albino male mice. The antibacterial activity was performed against Gram-positive and Gram-negative clinical strains by agar well diffusion method. The *in vitro* antiviral activity was carried out against camelpox and buffalopox viruses. The analgesic activity exhibited by the compounds 4a, 4c and 4d were found to be more significant compared to the standard. The bacterial activity was determined by the inhibition of growth of the organism by the drugs at different concentrations. All the compounds showed significant activity when compared with the drug ciprofloxacin. The *in vitro* antiviral activity of the compound 4b tested against camelpox and buffalopox viruses revealed no activity when tested at concentrations of 250 µg. The compound 4b did not alter the titres of both the viruses and the titres remain, respectively, 10<sup>6.5</sup> TCID<sub>50</sub> and 10<sup>6.74</sup> TCID<sub>50</sub> per ml for camelpox vaccine virus and buffalopox vaccine virus. However, the compounds 4a-e showed significant analgesic and antibacterial activities.

Key words: Analgesic, antibacterial, antiviral, benzo[f]chromen-3-ones-oxadiazoles, buffalopox virus, camelpox virus, photoluminescence,

Coumarin and its derivatives represent one of the most active classes of compounds possessing a wide spectrum of biological activity<sup>[1-9]</sup>. Many of these compounds were reported as antitumour<sup>[1]</sup>, antibacterial<sup>[2]</sup>, antifungal<sup>[3]</sup>, anticoagulant<sup>[4]</sup> and antiinflammatory<sup>[5]</sup> agents. In addition, these

compounds are being used as additives to food, cosmetics<sup>[6]</sup>, and fluorescent and in laser dye<sup>[7,8]</sup> applications. Since fluorescence is highly sensitive to physicochemical environments, a variety of organic fluorescent compounds (fluorophores) have been widely used as fluorescent labelling reagents<sup>[9]</sup> and fluorescence probes<sup>[10]</sup> in medical field. Thus, the coumarin nucleus has been the focus of recent

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investigation concerning the design of photoactive and luminescence probes<sup>[9]</sup>. Recently, the synthesis of various such novel photoluminescent benzo[*f*]chromen-3-ones-oxadiazoles (4a-e) has reported in our laboratory<sup>[10]</sup> (fig. 1). Since these molecules exhibit blue fluorescent properties; they are very useful in biomedical applications. Hence, as a preliminary study, we have screened these molecules for various biological activities such as analgesic, antibacterial and antiviral. The excellent light-emitting properties exhibited by benzo[*f*]chromen-3-ones-oxadiazoles (4a-e).

All solvents/chemicals used were of analytical grade and obtained from Merck, Mumbai, India. Eagle's minimum essential medium (EMEM) from GIBCO®, Invitrogen, USA. Fetal bovine serum (FBS) from Hyclone, Logan, USA and crystal violet from Qualigens, Glaxo SmithKline, Mumbai, India.

The experiments were carried out under sterile, aseptic conditions at 25±2°. The containers and instruments were sterilised using 5% formaldehyde and 90% ethanol, and dried before use. Gram-positive and Gram-negative clinical isolates of bacterial strains were collected and stored in an incubator at

37° for experimental use. Adult Swiss albino mice (25-30 g body weight) were obtained from the Viral Diagnostic Laboratory, Shivamogga, Karnataka, India. Institutional Animal Ethical Committee constituted by S. C. S. College of Pharmacy, Harapanahalli, Karnataka, India (Reg. no. 157/1999/CPCSEA) was given permission to conduct analgesic activity experiments on mice. In each group, six animals were housed individually in polypropylene cages with paddy husk bed. Animals were maintained at 25-27° and 30-70% relative humidity.

The analgesic activity was performed by acetic acid-induced writhing test in mice<sup>[11]</sup>. Male Swiss albino mice were obtained from Viral Diagnostic Laboratory, Shivamogga, Karnataka, India. Seven groups of six mice each (25-35 g of body weight) were selected and 0.6% acetic acid (dose 10 ml/kg of body weight) was injected intraperitoneally. The numbers of writhes were counted for 20 min, after 5 min of injection of acetic acid to each mouse, and this reading was taken as a control. The following day, the same groups of mice were used for evaluating analgesic activity. The five test groups 4a-e of benzo[*f*]chromen-3-ones-oxadiazoles suspension were administered orally to each group at a dose of 100 mg/kg body weight 1 h before injection of acetic acid. After 5 min of acetic acid injection, the mice were observed for the number of writhes for duration of 20 min. The mean value for each group was calculated and compared with that of control. Acetyl salicylic acid was used as a standard for comparison of analgesic activity. Mean value for each group was calculated and compared with control. Percent protection was calculated using the formula  $(1-V_t/V_c) \times 100$ . Where,  $V_t$  is mean number of writhing in test animals and  $V_c$  is mean number of writhing in control.

The antibacterial activity of all benzo[*f*]chromen-3-ones-oxadiazoles (4a-e) were tested by agar well diffusion method<sup>[9]</sup> against Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus haemolyticus*) and Gram-negative (*Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*) bacterial strains. The bacterial strains used for screening of the antimicrobial activity were collected from different infectious patients from Kiran Diagnostic Health Centre, Chitradurga (Karnataka State), India. The plates were incubated for 24 h at 37°. Ciprofloxacin

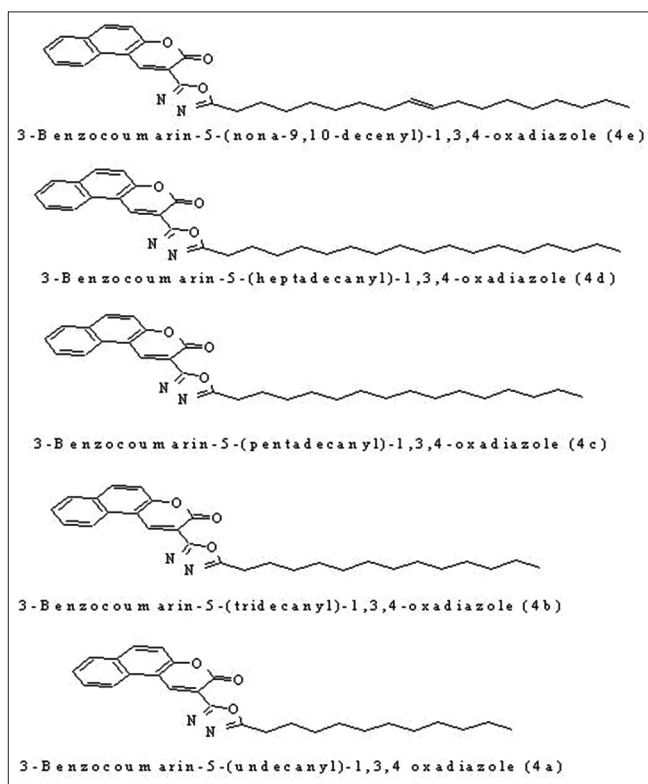


Fig. 1: Structures of 2-(5-alkyl-1,3,4-oxadiazol-2-yl)-3H-benzo[*f*]chromen-3-ones (4a-e)

(Sigma Chemical, St. Louis, Mo., USA) was used as the standard (50 µg in 100 µl of distilled water).

The antibacterial activity of all benzo[*f*]chromen-3-ones-oxadiazoles (4a-e) was determined by a micro dilution method in nutrient broth<sup>[10]</sup>. The inoculums were prepared using the nutrient broth at a density adjusted to a 0.5 McFarland turbidity standard colony forming units and diluted to 1:10. The plates were incubated at 37° and minimum inhibitory concentrations (MIC) was determined after 24 h of incubation. Sterilised nutrient medium was poured into sterile petri dishes. Nutrient broth containing 100 µl of 24 h incubated cultures and optical density (OD) was read at 660 nm. The respective clinical strain was spread separately on the agar medium. The wells were created using a stainless steel sterilised cork borer under aseptic conditions. The compounds at concentrations of 50 µg was dissolved in 100 µl of dimethyl sulphoxide (DMSO) and later loaded into the corresponding wells. The standard drug ciprofloxacin was used (50 µg in 100 µl). The plates were incubated for 24 h at 37° and the diameter of the zone of complete inhibition of bacteria was measured around each well and the readings were recorded. The results of these experiments were expressed as mean±SE of six replicates in each test. The data were evaluated by one-way ANOVA followed by Tukey's pair-wise comparison test Pair-wise comparison test and results were considered significant when  $P < 0.05$ .

The antiviral activity of these compounds was tested using camelpox and buffalopox viruses. The camelpox vaccine virus (attenuated camelpox1 isolate, passage 50) (CMLV-1, P-50) and buffalopox vaccine virus (attenuated Vijayawada 96 isolate, 50<sup>th</sup> passage) (BPXV, Vij/96, P-50) adapted to Vero cells was used as a source of the viruses. The Vero cell monolayer was infected with both viruses at 0.01 multiplicity of infection (m.o.i) and the virus allowed to adsorb on to cells at 37° for 1 h. Then, the monolayer was fed with maintenance medium and incubated for 2-3 days until >80% cytopathic effect (CPE) was observed with a change of medium on every alternative day. The virus was harvested when infected Vero cells showed virus-specific CPE characterised by rounding, ballooning, increased refractivity and detachment of Vero cells. After harvesting, the cells were freeze-thawed thrice and the virus was titrated in Vero cell monolayer. The titre was calculated as per Reed and Muench<sup>[12]</sup> and expressed as log tissue

culture infective dose 50% (TCID<sub>50</sub>). The titrated virus was stored in aliquots at -80° until further use.

The test compounds were dissolved in DMSO to obtain 3 mg/200 µl stock solution. Aliquots of the stock solution were kept at -20° and diluted in cell culture media right before use. The cytotoxicity of 4b compound to Vero cells was determined following the method described by Bhanuprakash *et al.*<sup>[13]</sup> with modifications. In brief, initially 4b compound dissolved in DMSO and different concentrations of compound with twofold dilutions were prepared in EMEM. Later, it was added to the 24 h confluent Vero cell monolayer in 96-well tissue culture plates and incubated in an atmosphere of 5% CO<sub>2</sub> at 37° for 4 days. Each concentration was tested in triplicate along with controls. Cells were examined daily for morphological changes (rounding, degeneration, cell lysis), if any. Cells were observed at every 24 h interval for visible morphological changes under inverted microscope (Leitz). Cell morphology was compared between treated and untreated cultures (control). The highest concentration of the 4b compound that showed no cellular morphological changes was considered as the maximum nontoxic concentration/dose (MNTC/MNTD). Further, at the end of the incubation period, the test medium was removed from the plate and washed gently with phosphate buffered saline (0.1 mol). The wells were stained with crystal violet (1%) in formalin (10% v/v) for 15 min. The cell viability was evaluated as the percentage of the mean value of OD resulting from the six cell controls, which was set at 100%. The 50% cytotoxic concentration (CC<sub>50</sub>) was calculated from the mean dose response of three independent assays. The concentration of compound at which cell viability was above 80% was further used in the study for assessment of antiviral activity of the 4b compound against CMLV and BPXV in a dose-dependent manner.

Antiviral activity was determined by reduction of virus titres using TCID<sub>50</sub> determinations<sup>[14]</sup>. Vero cells were grown in cell culture plates containing 96 wells for 48 h. After decanting the growth medium, the cells were treated with chemicals at their respective MNTCs (50 µl/well) and immediately logarithmic dilutions of virus (CMLV/BPXV) were added at 50 µl/well in treated and untreated cell cultures and incubated at 37° in CO<sub>2</sub> (5%) for 24 h. After 24 h of simultaneous incubation, both chemical and the virus were discarded and the wells were replenished with

fresh maintenance medium. Subsequent change of medium was done regularly at 48 h intervals and the virus titres (TCID<sub>50</sub>/ml) were calculated after 6 days<sup>[3]</sup>. The experiment was carried out in triplicates. The antiviral activity was expressed as percentage of inhibition (PI) using antilogarithm values of TCID<sub>50</sub>, as: PI=[1-(T antilogarithm/C antilogarithm)]×100. A chemical is considered active when the PI is >80% at its MNTC

Analgesic activity of benzo[f]chromen-3-ones-oxadiazoles (4a-e) was carried out using acetic acid-induced writhing test. This experiment results yielded significant values for synthetic compounds (4a-e) compared to standard and control values (Table 1).

The benzo[f]chromen-3-ones-oxadiazoles (4a-e) showed significant antibacterial activity against Gram-positive and Gram-negative bacteria. The significant value of antibacterial activity was exhibited by test compounds and was comparable to the drug ciprofloxacin (50 µg in 100 µl) and control (Table 2). The compounds benzo[f]chromen-3-ones-oxadiazoles

(4a-e) were found to be active against the set of organisms at a concentration of 50 µg/100 µl DMSO.

Cytotoxicity of 4b compound on host cells (Vero cells from an African green monkey kidney cell line) was studied. The MNTC/MNTD of 4b compound was found at a concentration of 250 µg/ml at which there was no visible toxicity to the cells. The Vero cells tolerated MNTCs of the compound 4b. Hence, the concentrations below MNTCs were utilised for antiviral efficiency testing. When the 4b compound was tested for its antiviral activity below its MNTC against CMLV and BPXV, it failed to inhibit the replication of these viruses *in vitro*. The titres of both the viruses remained 10<sup>6.5</sup> and 10<sup>6.74</sup>/ml, respectively, both in control and treated wells. This indicates that the 4b compound is noninhibitory/avirucidal (data not shown) against these viruses; rather, infected and treated cells showed virus-specific CPEs.

In the present investigation, the synthesised fluorescent compound series of 2-(5-alkyl-1,3,4-oxadiazol-2-yl)-3*H*-benzo[f]chromen-3-ones (4a-e) have been evaluated for antibacterial, analgesic and antiviral activities. The analgesic activity exhibited by the compounds 4a, 4c and 4d was found to be more significant compared to the standard acetyl salicylic acid by inducing acetic acid-writhing method in mice. This acetic acid-induced writhing response was evident for peripheral activity; acetic acid-caused algia by producing endogenous substances such as serotonin, histamine and prostaglandins, thereby stimulating pain in nerve cells<sup>[15]</sup>. This in turn causes abdominal writhing responses<sup>[16]</sup>. Thus, the experimental method involved in association of increased level of prostaglandins (PGE<sub>2</sub> and PGE<sub>2α</sub>) in peritoneal fluid and increased levels of lipoygenase metabolites<sup>[17]</sup>. Hence; the synthesised compounds may act by suppressing the synthesis or release of above stated endogenous substances. From the above facts

**TABLE 1: ANALGESIC ACTIVITY OF BENZO[F]CHROMEN-3-ONES OXADIAZOLES (4A-E)**

Compounds	Dose mg/kg	Mean no. of writhing		Protection (%)
		Before administration of drug (mean±SE)	After administration of drug	
4a	100	34.5±46.283	16.33±0.49	52.66*
4b	100	34.16±0.65	16.83±0.5715	50.73
4c	100	35.33±0.577	16.33±0.517	53.77*
4d	100	35.5±0.577	16.16±0.605	54.47*
4e	100	23.00±2.89	11.16±1.07	51.45
Standard	100	34.66±0.93	16.66±0.44	52.00
Control	100	33.66±0.816	33.33±1.0	01

Values are in mean±SEM. Index for analgesic activity, Method=Acetic acid induced writhing (acetic acid-0.6%), Animal=Swiss albino male mice, Number of animals per group: 6 (25-30 g each), Route of administration=Intraperitoneally (IP), Standard drug used=Acetyl salicylic acid (Aspirin), SEM=Standard error mean, \* = P<0.05

**TABLE 2: ANTIBACTERIAL ACTIVITY OF BENZO[F]CHROMEN-3-ONES OXADIAZOLES (4A-E)**

Compound	<i>Streptococcus haemolyticus</i>	<i>Staphylococcus aureus</i>	<i>Salmonella typhi</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Klebsiella pneumoniae</i>
4a	21.00±0.58	23.00±1.53	24.67±2.40	24.67±1.76	21.33±0.88	24.67±1.86	22.67±0.88
4b	22.67±1.76	23.33±1.76	25.00±0.58	22.67±0.88	24.00±2.31	24.00±0.58	21.00±0.58
4c	22.33±0.88	22.67±1.45	24.33±2.03	25.00±0.58	23.67±1.76	25.00±2.52	23.00±1.15
4d	24.00±2.31	21.67±1.76	25.00±2.31	21.67±1.20	21.00±2.00	22.33±0.88	22.00±1.15
4e	20.67±0.88	23.00±1.15	21.67±1.20	22.67±2.19	21.00±1.53	22.67±0.88	24.67±2.85
Standard	23.67±0.33	24.00±0.58	26.67±1.76	27.00±1.73	27.33±1.45	25.67±2.03	27.00±2.52
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0

The value of each constituents consisted of mean±SEM n=03. Value significantly different when P<0.05. Standard drug used=Ciprofloxacin. (50 µg in 100 µl), Control=Dimethyl sulphoxide (DMSO), Drug used=(40 µg in 100 µl)

and experiments, it can be observed that percentage of protection is significant for the synthesised compounds (4a-e) compared to standard and control groups (Table 1). Antibacterial activity of the compounds was determined by agar well diffusion method using ciprofloxacin as a standard. The data of these synthesised compounds showed significant values compared to standard and control (Table 2). Bacteria have negatively charged surface structures, such as lipopolysaccharides or lipoteichoic acids, and their membranes contain negatively charged phospholipid, such as phosphatidylglycerol. On the other hand, eukaryotic cells such as erythrocytes contain zwitter ionic phosphatidylcholine. Therefore, the antimicrobial compounds with positive charge selectively bind to the outer leaflet of the bacterial membrane rather than the eukaryotic membrane *via* the electrostatic interaction. Furthermore, we have investigated the compound 4b for antipoxvirus studies. The compound 4b used in the test was dissolved in DMSO. Although other cell lines such as primary chick embryo, chick kidney, calf kidney, Madin Darby Canine Kidney (MDCK), mink lung and human respiratory epithelial cells may be used for *in vitro* antiviral assays<sup>[18]</sup>, Vero cells were used in the present study because these viruses were adapted to grow this cell line. We tested the effect of various concentrations of DMSO on Vero cell monolayers by dye exclusion method assay, resulting in near to 100% cell survival at less than 1% DMSO after 48 h incubation (data not shown). Moreover, potential interference of DMSO in the assay throughout the screening process was assessed for every plate with the addition of 0.2 µl of DMSO to three wells, volume equivalent to that one of the compounds. Selection of CMLV and BPXV inhibitors may be of value in obtaining antiviral agents against pox viruses<sup>[19]</sup> in general and smallpox virus in particular, which has reemerged as a threat for use as a bioterrorism agent<sup>[20]</sup>. The investigation on the compound 4b showed that it has no inhibitory effect on two pox virus (DNA virus) viz. CMLV and BPXV *in vitro*. However, search for antiviral activity with other viruses is warranted, considering the scarcity of the number of antiviral agents.

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