RESEARCH ARTICLE

THE EFFECTS OF NEWLY SYNTHESIZED XANTHENE-3-ONES ON *PSEUDOMONAS AERUGINOSA* – EXPERIMENTAL STUDY ON RATS

Alisa Smajović[1*], Elma Veljović[2], Mirza Čelebičić[3]

[1]Department of Social Pharmacy and Pharmaceutical Legislation, University of Sarajevo – Faculty of Pharmacy, Sarajevo, Bosnia and Herzegovina

[2]Department of Pharmaceutical Chemistry, University of Sarajevo – Faculty of Pharmacy, Sarajevo, Bosnia and Herzegovina

[3]Independent researcher, Sarajevo, Bosnia and Herzegovina

[*]**Corresponding author:**
Asst. Prof. dr. sc. Alisa Smajović
Department of Social Pharmacy and Pharmaceutical Legislation, University of Sarajevo
Sarajevo/Bosnia and Herzegovina

Address:
Zmaja od Bosne 8
71000 Sarajevo

Phone: +387 33 586 191
ORCID: 0000-0002-2494-6744
E-mail: alisa.smajovic@ffsa.unsa.ba

Original Submission:
22 February 2023
Revised Submission:
04 March 2023
Accepted:
24 March 2023

How to cite this article: Smajović A, Veljović E, Čelebičić M. 2023.The effects of newly synthesized xanthene-3-ones on *Pseudomonas aeruginosa* – Experimental study on rats. Veterinaria, 72(2), 153-163.

ABSTRACT

The need for new drugs that will have an antimicrobial effect is increasing every day. Xanthenes are cyclic, organic compounds of natural, semi-synthetic or synthetic origin, which in many studies have shown good antimicrobial, antioxidant, antiproliferative, antidiabetic, neuroprotective and many other biological effects and are interesting for further research. In the study, the antimicrobial activity of two newly synthesized xanthenic compounds was investigated: 2,6,7-trihydroxy-9-(2-hydroxy-5-bromophenyl)-3H-xanthene-3-one (Compound 1) and 2,6,7-trihydroxy-9-(3-bromophenyl)-3H-xanthene-3-one (Compound 2) to a wound infection caused by a bacterial strain *Pseudomonas aeruginosa* (ATCC 10145) on rats (n=36). The animals were divided into 6 groups. The first group was treated with Compound 1 in the concentration of 0,626 mg/g, the second group with Compound 1 in the concentration of 1 mg/g, the third with Compound 2 in the concentration of 0,626 mg/g and the fourth by Compound 2 in the concentration of 1 mg/g. The fifth and sixth groups were comparative (gentamicin) and control (vaseline). The Compounds were prepared in the form of a dermal preparation with the concentrations of 0,626 mg/g and 1 mg/g and were applied to the wound every day in the amount of 1 mg. Swabs of the wounds were taken on 1., 2., 3., and 7. day after infection to determine whether there has been a reduction in the number of bacteria. The results showed that both Compounds ultimately led to the subsidence of the infection, but a statistically significant difference was only observed between the groups that were treated with Compound 2 in the concentration of 0,626 mg/g compared to the control group.

Keywords: Antimicrobial effect, Xanthenes, *Pseudomonas aeruginosa*

doi.org/10.51607/22331360.2023.72.2.153

INTRODUCTION

The problem of resistance in microorganisms to already existing antimicrobial drugs has long been alarming all over the world. Already known antibiotics, which have been in use for many years, but also some newer ones, are increasingly becoming ineffective in the fight against bacteria. Various studies indicate that inadequate prescribing and use of antibiotics (Davey et al., 2017) as well as the fact that the pharmaceutical industry is facing great challenges, economic and regulatory when it comes to the development of new drugs, contribute to this problem (Gross, 2013; Bartlett et al., 2013; Sengupta et al., 2013; Wright, 2014; Viswanathan, 2014; Read and Woods, 2014; Lushniak, 2014; Michael, et al., 2014). From a medical point of view, the result is that the therapeutic effect of antibiotics is disabled, that is, the patient's recovery is missing, and from an economic point of view, the costs of the patient's treatment increase due to the patient's longer stay in the hospital and longer recovery (Lushniak, 2014). The development of new drugs is a long-term and expensive process, and, considering the aforementioned resistance as well as the appearance of new pathogenic strains, there is a constant need to find new pharmacologically active compounds that will be used in human and animal therapy. In order to rationalize the time and money invested in the discovery and synthesis of new drugs, the attention is paid to the isolation and semi-synthetic production of compounds from natural materials so that often on the basis of knowledge obtained from the isolates, the synthesis of completely new compounds is also approached (Mishra and Tiwari, 2011).

One such group of compounds is xanthenes and their derivatives. It is a special group of tricyclic compounds that contain oxygen, characterized by a pyranheterocycle that contributes to their reactivity (Maia et al., 2021). Although they can also be found in nature (Imran et al., 2017; Robertson et al., 2019), a large number of xanthene compounds are of synthetic origin (Veljović et al., 2015; Kamat et al., 2021). Many toxicity tests show that xanthene compounds have no toxic effect in the tested concentrations. So, in the study of Reddeman et al. (2019), it was determined that natural xanthene mangiferin, when administered orally to rats, does not show toxic effects up to a concentration of 2000 mg/kg bw/day, while in the study of Smajović et al. (2020), the toxic effect of synthesized xanthenes was investigated after parenteral administration, and no histopathological changes were observed in rats. Even at the molecular level, potential cytotoxicity and genotoxicity were not observed (Veljović et al., 2019).

Xanthene compounds show a very wide spectrum of biological activities. In a rat study that was performed by Epstein et al. (2014), neuroprotective action has been proven, while Manikandan et al. (2020), also in animal model, proved the antitumor activity of xanthene derivatives. Various *in vitro* studies show that xanthenes have an antitumor effect on human cell lines of various tumors, such as prostate, colon, lung and leukemia tumors (Giri et al., 2010). Anti-inflammatory (Banerjee et al., 2016), antiparasitic (Wu et al., 2005), antidiabetic (Irondi et al., 2016; Patarakijavanich et al., 2019), antioxidant (Veljović et al., 2015), and antifungal (Yunnikova et al., 2013) are just some of the pharmacological effects that xanthenes and their derivates, whether of natural or synthetic origin, exhibit. What is of special interest for our study is the current knowledge about the antimicrobial activity of xanthene derivatives (Veljović 2018; Zukić 2018), and, especially, the tests of effects on *Pseudomonas aeruginosa*, where xanthene derivatives have shown good results (Amininasab et al., 2020a; Amininasab et al, 2020b).

Pseudomonas aeruginosa is a gram-negative bacterium that is responsible for a wide range of serious acute and chronic diseases (Bitsori, 2012; Sousa and Pereira, 2014; Mayer-Hamblett, 2015; Winstanley et al., 2016; Lin et al., 2016; Newman, 2017; Diekema et al., 2019; Fabre et al., 2019; Montero et al., 2020). Also, open wounds in humans and animals are very susceptible to infection with this bacterium. Some studies even show that the wounds induced by *P. aeruginosa* are much bigger than some others caused by other

types of bacteria such as *Staphylococcus aureus*, which may be an indication that *P. aeruginosa* infection can be more detrimental to the wound healing process than when the infection is caused by another causative agents of the disease (Mendes et al., 2012). Antibiotics (e.g. beta lactams, fluoroquinolones, aminoglycosides), which are used in therapy alone, or in combination are becoming increasingly ineffective, bearing in mind that the rate of resistance of clinical isolates of *P. aeruginosa* has increased sharply in the last 5 decades (Zhang et al., 2015; Lynch et al., 2017), which is noticeable if the infection is caused by multidrug resistance (MDR) *P.aeruginosa* strains (Karlowsky et al., 2005; Nathwani et al., 2014; Ciofi Degli Atti et al., 2014; Potron et al., 2015; Carmeli et al., 2016).

The aim of this study is to examine the antibacterial effect of newly synthesized xanthenes:2,6,7-trihydroxy-9-(2-hydroxy-5-bromophenyl)-3H-xanthene-3-one (COMPOUND 1) and 2,6,7-trihydroxy-9-(3-bromophenyl)-3H-xanthene-3-one (COMPOUND 2) on wound infection caused by bacterial strain *Pseudomonas aeruginosa* (ATCC 10145) so that they could eventually be used as potential antibiotics in human and veterinary medicine.

MATERIAL AND METHODS

Compounds

Tested compounds: 2,6,7-trihydroxy-9-(2-hydroxy-5-bromophenyl)-3H-xanthene-3-one (COMPOUND 1) i 2,6,7-trihydroxy-9-(3-bromophenyl)-3H-xanthene-3-one (COMPOUND 2) were synthesized at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Sarajevo. The composition and structure of the tested compounds were confirmed by elemental analysis, infrared spectroscopy, nuclear magnetic resonance (1H-NMR i 13C-NMR), mass spectrometry and crystallographic analysis (Veljović et al., 2015; Applova et al., 2017).

The dermal preparations the animals were treated with were prepared at the Department of Drug Formulation, University of Sarajevo - Faculty of Pharmacy. The preparation of Compound 1 with the concentration of 0,626 mg/g was prepared by weighing a mass of 6,26 mg of Compound 1 and 10 g of vaseline on an analytical scale. Both ingredients were transferred to a mortar with a pestle and mixed until a uniform mass was obtained. In this way, 10 g of dermal preparation of Compound 1 with the concentration of 0,626 mg/g was prepared.

The same procedure was carried out for the other preparations, taking into account the type of compound and the concentration to be achieved.

Animals

As animal models in the study were used both gender rats, Wistar strain, aged 2-3 months, average body weight 180-250 g (n=36). The rats had free access to food and water during the experiment and a 12-hour shift of light and darkness. The air temperature ranged between 20 and 23ºC, and humidity 60% ± 10%. The animals were divided into 6 groups depending on which treatment was used: the first group (n=6), Compound 1 in the concentration of 0,626 mg/g; the second group (n=6), Compound 1 in the concentration of 1mg/g; the third group (n=6) Compound 2 in the concentration of 0,626 mg/g; the fourth group (n=6), Compound 2 in the concentration of 1mg/g; the fifth group (n=3), comparative group with gentamicin ointment (GENTAMICIN BOSNALIJEK 1mg/g) and the sixth group (n=9), control group with vaseline (Vazelin Ph. Eur. 8.0., Semikem).

Preparation of inoculum

Bacterial strain *Pseudomonas aeruginosa* (ATCC 10145) was plated on agar, then left in a thermostat at 37ºC for 24h. After the culture was developed, a part of the colonies from the Petri dish was captured by inoculation loop and transferred to a test tube with 0,9% NaCl. In order to obtain the desired concentration of 10^6 CFU/ml, the turbidimetric method was used, in which the turbidity of the solution is monitored and the concentration is read on the turbidimeter.

Preparation of nutrient media

As a nutrient medium was used Pseudomonas Agar Base prepared according to the standard procedure. Petri dishes were previously sterilized for 15 minutes at 121ºC in an autoclave, and then cooled to 45-50ºC. The prepared medium was then poured into Petri dishes in a layer of 2-5mm. After they were completely cooled, the substrates were used for sowing.

Experimental procedure

At the beginning of the experimental procedure, all tested animals were acclimatized and then put under general anesthesia using 5 mg/kg xylazine hydrochloride 2% (2% Xylazin, CpPharma, Bergdorf, Germany) and 50 mg/kg ketamine hydrochloride (Ketamine HCl Injection USP, Rotexmedica, Germany) intramuscularly. After the hair covering was removed and the skin was disinfected with povidone-iodine solution (Povidonjod HF 10%, HEMOFARM d.o.o. Banja Luka), a 1-1,5 cm long incision was made on the back, taking care to make a full incision. The previously prepared inoculum was carefully instilled into the wound in a volume of 0,25 ml, after which the sterile gauze was placed on the wound, and the animals were returned to the cage. After 24 hours, the first swab of the wound was taken to determine whether an infection had developed.

After taking the swab, each animal was treated with 1g of the preparation, depending on the group it was in. Then the sterile gauze was placed on the wound, and the procedure of applying the preparation was repeated every day at the same time. The swabs were taken on the second, third and seventh day of the experiment. Then, they were seeded on a nutrient medium, left in a thermostat for 24 hours at a temperature of 37ºC, after which the colonies were counted using the Koch method.

Statistical data processing

The statistical data processing was done in the program SPSS 24.0 (IBM Corp., 2016). Tests that are used were ANOVA and MIXANOVA, with post hoc tests, and the difference at the $p<0,05$ level was considered statistically significant.

Approval of the Ethics Committee

Approval for the experimental work was issued by the Ethics Committee of the Veterinary Faculty, University of Sarajevo, under number 01-02-18-12/18.

RESULTS

36 rats were utilized in the investigation and were split into two groups: the first (n=18) treated with Compound 1 in the concentrations of 0,626 mg/g and 1 mg/g, gentamicin and vaseline as comparative, i.e. control group (Table 1) and the second group (n=18) treated with Compound 2 in the concentrations of 0,626 mg/g and 1 mg/g, gentamicin and vaseline as comparative, i.e. control group (Table 2). As can be seen in both tables, the swab on Day 1 showed in all rats, except for two, the concentration of bacteria of 10^6CFU/ml, which is a confirmation of the presence of infection. A decrease in the number of bacteria is observed after the 2nd day, while from the 3rd day, in some rats whose wounds were treated with Compound 1, the swabs were sterile, that is, in some rats whose wounds were treated with Compound 2, the wounds were sterile after the 7th day of infection.

Table 1 The number of bacteria in rats after the administration of Compound 1

	Compound 1 concentration 0,626 mg/g						Compound 1 concentration 1,0 mg/g						Gentamicin (comparative group)			Vaseline (control group)		
rat	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
1.day	10^6	10^5	10^5	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6
2.day	10^6	360	300	10^6	10^6	10^6	10^6	10^5	4176	S	10^4	10^6	10^5	10^6	10^6	10^5	10^5	10^5
3.day	10^6	S	S	10^4	10^4	10^5	10^6	10^3	28	S	126	10^4	4	160	120	10^4	10^4	10^4
7.day	10^4	S	S	10^2	10^2	10^3	412	568	S	S	20	292	S	136	20	6272	440	10^3

S –sterile

Table 2 The number of bacteria in rats after the administration of Compound 2

	Compound 2 concentration 0,626 mg/g						Compound 2 concentration 1,0 mg/g						Gentamicin (comparative group)			Vaseline (control group)		
rat	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
1.day	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6
2.day	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^5	10^6	10^6	10^5	10^6	10^5	10^6	10^6	10^6	10^6	10^6
3.day	10^5	464	10^5	10^5	10^5	10^5	2480	10^4	10^3	10^5	1200	10^5	380	10^3	800	10^5	10^4	10^4
7.day	1976	S	10^3	5760	S	1712	S	9600	112	120	168	10^3	S	256	S	10^3	1560	1440

S –sterile

If we compare these two compounds, based on the statistical processing of the data, no statistically significant difference was observed if the time required to reduce the number of bacteria was observed, but if the action itself was observed, the only statistically significant difference was

observed between the groups of rats that were given Compound 2 in the concentration of 0,626 mg/g and control group that received vaseline (p=0,009, p<0,05). All other groups did not differ statistically (Table 3).

Table 3 Effect of Compounds 1 and 2 on *Pseudomonas aeruginosa*

Tested compounds*	p value (time)	p value (compounds)
1A-1B	$4,68*10^{-7}$	0,12
2A-2B	$7,9*10^{-8}$	0,352
1A-2A	$2,23*10^{-7}$	0,09
1B-2B	$4*10^{-6}$	0,192
1A-Gentamicin	$1,1*10^{-5}$	0,452
1B-Gentamicin	$1,98*10^{-4}$	0,327
1A-Vaseline	0,001	0,06
1B-Vaseline	$5,4*10^{-5}$	0,571
2A-Gentamicin	$4*10^{-6}$	0,416
2B-Gentamicin	0,001	0,922
2A-Vaseline	$1,19*10^{-4}$	0,009**
2B-Vaseline	$2,33*10^{-4}$	0,067

*A=0,626mg/g; B=1,0 mg/g;
1=Compound 1;2=Compound
** statistically significant

DISCUSSION AND CONCLUSION

P. aeruginosa is a bacterium that cancels the effect of antibiotics by different mechanisms so that the resistance develops very easily. In 2017, the World Health Organization recognized this problem, and put *Pseudomonas aeruginosa* on the list of priorities for research and development of new antibiotics that will overcome the increasingly common problem of resistance (WHO, 2017).

The skin is an organ that represents a barrier against various types of microorganisms. As long as it is not damaged in some part, *Pseudomonas aeruginosa* cannot cause an infection (Coates et al., 2018). However, if a suitable environment is developed, *Pseudomonas aeruginosa* will very quickly form colonies and develop an infection (Tosh et al., 2011). on which basis the design of this experiment was developed.

In this study, all rats were infected with 10^6CFU/ml (except two rats), and from the obtained results it can be observed that already on the 2nd day after the infection, Compound 1 in the concentration of 0,626 mg/g also worked in two rats, while the number of bacteria in the swabs was 360, i.e. 300. Even better result is observed in the group treated with Compound 1 in the concentration of 1 mg/g, where after the 2nd day in one rat the swab was sterile, and in other the number of bacteria was 4176. If we compare this with the effect of Compound 2 in both concentrations, we will see that there were no changes in the number of bacteria after the 2nd day. Also, after the 2nd day, there were no changes in either the comparative or the control group. Only on the 3rd day after infection, the changes are observed in all tested groups. In the group in which Compound 1 was used in the concentration of 0,626 mg/g, the wounds of two rats were sterile, while in the group where Compound 1 was used in the concentration of 1 mg/g, the number of rats with a sterile wound remained the same, but a significant decrease in the number of bacteria was observed in other rats from the group. Also, in the comparative group, the number of bacteria is significantly lower compared to the previous swabs, so that the number is below 200. After the 3rd day, in the groups in which Compound 2 was applied in both concentrations, a slight decrease in the number of bacteria was observed, but still not a single rat had a sterile wound. After the 7th day of infection, three rats had the sterile wounds, which were treated with Compound 2 in the concentration of 0,626 mg/g and one tested Compound 2 in the concentration of 1mg/g. After taking the last swab in the group to which Compound 1 was applied, four rats had a sterile wound, two from the group to which Compound 1 was applied in concentration of 0,626 mg/g and two from the group to which Compound 1 was applied in the concentration of

1mg/g.

Bearing in mind the time period that should have passed to obtain sterile wounds in at least one animal, we can conclude that Compound 1 has a better antimicrobial effect than Compound 2.

However, by statistical processing of the data (Table 3), the only statistically significant difference was observed between the groups of rats that were administered Compound 2 compared to the control group ($p<0,05$), but we should not ignore the results between the groups to which Compound 1 was applied in the concentrations of 0,626 mg/g and 1 mg/g ($p=0,09$), groups that were administered Compound 1 in the concentration of 0,626 mg/g and vaseline ($p=0,06$), as well as between the groups that were administered Compound 2 in the concentration of 1 mg/g and vaseline ($p=0,067$).

From this, it can be concluded that the results of our test indicate that the compounds have a potential antimicrobial effect. Taking into account the length of the test time and the decrease in the number of bacteria during that time, the conclusion is that, most likely, a more realistic picture of the antimicrobial effect of the compounds would be obtained if the treatment time of the infected wounds were extended, while considering an increase in the concentration of the tested compounds, as well as testing a larger number of differently substituted xanthene derivatives. In accordance with the above, further research is necessary.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

CONTRIBUTIONS

AS: 1,2,3,4,5,6,7,8,9,10; EV: 1,8 10; MČ: 1,2,6,10.

Concept (1); Design (2); Supervision (3); Resources (4); Materials (5); Data Collection and/or Processing (6); Analysis and/or Interpretation (7); Literature Search (8); Writing Manuscript (9); Critical Review (10)

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EFEKTI NOVOSINTETIZIRANIH KSANTEN-3-JEDAN NA *PSEUDOMONAS AERUGINOSA* – EKSPERIMENTALNA STUDIJA NA STAHORIMA

SAŽETAK

Potreba za novim lijekovima koji imaju antimikrobno dejstvo je svakim danom sve veća. Ksanteni su ciklični organski spojevi koji mogu biti prirodnog, polusintetskog ili sintetskog porijekla i koji su u mnogobrojnim studijama pokazali dobro antimikrobno, antioksidantno, antiproliferativno, antidijabetično, neuroprotektivno i drugo biološko djelovanje te koji su od interesa za daljnje istraživanje. U ovim istraživanju je ispitivano antimikrobno dejstvo dva novosintetizirana ksantenska spoja: 2,6,7-trihidroksi-9-(2- hidroksi-5-bromofenil)-3H-ksanten-3-jedan (SPOJ 1) i 2,6,7-trihidroksi-9-(-(3-bromofenil)-3H-ksanten -3-jedan (SPOJ 2) kod infekcije rane izazvane bakterijskim sojem *Pseudomonas aeruginose* (ATCC 10145) na pacovima (n=36). Životinje su podijeljene u šest grupa. Prva grupa je tretirana sa spojem 1 u koncentraciji od 0,626 mg/g, druga grupa sa spojem 1 u koncentraciji od 1 mg/g, treća sa spojem 2 u koncentraciji od 0,626 mg/g i četvrta sa spojem 2 u koncentraciji od 1 mg/g. Peta i šesta grupa su komparativna (gentamicin) i kontrolna (vazelin). Spojevi su pripremljeni u obliku dermalnog preparata u koncentraciji od 0,626 mg/g i 1 mg/g koji su aplicirani na ranu svaki dan u količini od 1 mg.

1, 2, 3. i 7. dana nakon infekcije su uzimani brisevi rana kako bi se odredilo da li je došlo do smanjenja broja bakterija. Rezultati su pokazali da su u konačnici oba spoja dovela do povlačenja infekcije, ali je statistički signifikantna razlika uočena samo između grupa koje su tretirane sa spojem 2 u koncentraciji od 0,626 mg/g u usporedbi sa kontrolnom grupom.

Ključne riječi: Antimikrobni efekt, ksanteni, *Pseudomonas aeruginosa*