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Biological Evaluation of (2-(2-(2-Nitrophenyl) Diazinyl) Malononitrile): Focus on Antibacterial Activity

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التقييم البيولوجي لمركب (2-(2-(2-نيتروفينيل) داي ازينيل) مالونونيتريل مع التركير على النشاط المضاد للبكتيريا

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Abstract

The rise of multidrug-resistant (MDR) bacteria poses a significant challenge to the antibacterial efficacy of the produced chemical, (2-(2-(2-nitrophenyl) diazinyl) malononitrile), which is generated from malononitrile and a diazo moiety. The molecule was synthesized using the diazotization of 2-nitroaniline, followed by coupling with malononitrile, resulting in NPDAM with an 85% yield and a melting point of 123-125 °C. The substance at concentrations of 1, 5, and 10 mg/mL was assessed against methicillin-resistant Staphylococcus aureus (MRSA) (SA121) and Escherichia coli (EC49). The chemical exhibited efficacy against MRSA at varying doses, yielding clear zones of 29.6, 32.6, and 33.0 mm, in contrast to E. coli at the same concentrations, which produced clear zones of 10, 11.6, and 14.6 mm, respectively. The microdilution technique was employed to ascertain the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. The compound exhibited low minimum inhibitory concentration (MIC) values against MRSA at concentrations of 1 and 5 mg/mL (19.5 and 4.89 µg/mL, respectively), in contrast to E. coli, which demonstrated inhibition at 39.0 and 9.78 µg/mL at the same concentrations. Additionally, it yielded the lowest MIC values (0.97 µg/mL) against both bacteria at a concentration of 10 mg/mL. The MBC values against MRSA ranged from 39.0 to 1.95 µg/mL, while against E. coli, the MBC values were slightly lower, ranging from 78.1 to 1.95 µg/mL. In conclusion, all chemical concentrations exhibited activity against the studied microorganisms, indicating their potential as antimicrobial agents.

Keywords: Malononitrile, Diazo compounds, antimicrobial activity, MRSA, *E. coli*.

الملخص

يمثل ظهور البكتيريا المقاومة للأدوية المتعددة تحديا كبيرا للعلاج المضاد للميكروبات في هذا البحث تم تقييم النشاط المضاد للبكتيريا للمركب (2-(2-(2-نيتروفينيل) داي ازينيل) مالونونيتريل المعتمد في تركيبه على المالونونبتريل ومجموعة الديازو. تم تحضير المركب عن طريق دايازوتيزيشن لل 2- نيتروانيلين تلاه اقتران مع مالونونيتريل وأعطى المركب نسبة عائد 85% ونقطة انصهار 123-125 درجة مئوية. تم اختبار المركب عند تراكيز 1و5و 10 ملجم/مل ضد *Staphylococcus aureus* المقاومة للميثيسللين (MRSA) و *Escherichia coli*. أظهر هذا المركب بتركيزات مختلفة تأثيرا قويا ضد بكتيريا MRSA بمناطق تأثير بلغت 29.6 و32.6 و33.0 ملم على التوالي، مقارنةً ببكتيريا *E. coli* بنفس

التركيزات وبمناطق تأثير بلغت 10 و11.6 و14.6 ملم على التوالي. استُخدمت ايضا طريقة التخفيف الدقيق لتحديد قيم الحد الأدنى للتركيز المثبط والقاتل (MIC وMBC) ضد نوعي البكتيريا. وقد أظهر هذا المركب أدنى قيم تثبيط ضد MRSA عند تركيزات 1 و5 ملغم/مل حيث بلغت 19.5 و4.89 ميكروغرام/مل على التوالي، مقارنةً ببكتيريا *E. coli* التي ثُبطت بقيم 39.0 و9.78 ميكروغرام/مل على التوالي عند نفس التركيزات، وأعطى هذا المركب أدنى قيم مثبطة بلغت 0.97 ميكروغرام/مل ضد كلتا البكتريا عند تركيز 10 ملغم/مل. بشكل عام، تراوحت قيم التأثير القاتل بين 39.0 و1.95 ميكروغرام/مل عند تركيزات مختلفة ضد MRSA، بينما كان تأثيره القاتل الحيوي أقل بقليل ضد *E. coli*، حيث تراوحت قيم التأثير القاتل بين 78.1 و1.95 ميكروغرام/مل. في الختام، أظهرت جميع تركيزات المركبات نشاطًا ضد نوعي البكتيريا المختبرة، مما يدل على فعاليته كمضاد للميكروبات والتي قد تستخدم مستقبلا كعلاج جديد ضد أنواع من البكتيريا الممرضة.

الكلمات المفتاحية: مالونونيتريل، مركبات الدايازو، النشاط المضاد للميكروبات، MRSA، *E. coli*.

Introduction

A wide range of infections have become difficult to treat due to the rise of multidrug-resistant (MDR) pathogens including bacteria.

As a result, there has been a resurgence of interest in exploring underutilized sources of antimicrobial compounds, including secondary metabolites derived from medicinal plants as well as synthetic materials [1,2].

In medicinal chemistry, an important goal is to synthesize bioactive compounds that can be used as therapeutic agents with minimal side effects. As a result of the growing problem of antimicrobial resistance, many antibiotics have become less effective [4,5]. Accordingly, the rise of antibiotic-resistant infections has prompted researchers to seek alternatives with improved biological activity to combat these challenges [6].

Malononitrile is an extremely versatile chemical building block due to its distinctive reactivity, which makes it useful in a variety of applications in medicine, agriculture, and industry [7]. It has been employed in numerous chemical processes, such as a medium for polyacrylonitrile systems [8–10], acting as a desiccant for broad-leaf crops [11], and engaging in various polymerization reactions [12,13]. Moreover, malononitrile facilitates the synthesis of azomethine dyes and plays a role in dyeing polyacrylonitrile fibers.

In addition to its industrial use, malononitrile has been documented to provide protection against nitrogen-mustard toxicity and X-ray exposure [14–16], and previous research has highlighted its significance in poliomyelitis studies [19,20]. Various malononitrile compounds demonstrate significant pharmacological properties, including anticonvulsant and analgesic actions [11]. Moreover, it has served as a crucial precursor in the synthesis of 2-amino-4H-chromene compounds, which exhibit a wide range of biological actions, including antiproliferative, neurological, antimicrobial, anticancer, antiviral, antitumor, antimutagenic, and hormone-related properties [21]. This paper presents an efficient biologically active molecule derived from malononitrile and diazo moiety, focusing on the evaluation of its antibacterial activity as part of our ongoing studies on the biological activity of (2-(2-(2-nitrophenyl) diazinyl) malononitrile).

Material and methods

Synthesis of 2-(2-(2-nitrophenyl)diazenyl)malononitrile

The chemical 2-(2-(2-nitrophenyl) diazenyl) malononitrile was produced according to a previously documented method (H. H. Nawar, 2021). The aromatic amine precursor was transformed into its respective diazonium salt under cold acidic conditions. The intermediate was subsequently combined with malononitrile in an ethanolic medium, utilizing a buffering agent, resulting in the synthesis of the desired diazo–malononitrile derivative. The resultant solid was refined via recrystallization from ethanol, producing light brown crystals with a melting point of 123–125 °C and an overall yield of around 85% [22]. The overall synthetic route is depicted in Scheme 1.

Preparation of Bacterial Inoculum

The bacterial isolates, methicillin-resistant Staphylococcus aureus (MRSA SA121) and Escherichia coli (E. coli EC49), were acquired from the Biotechnology Research Center (BRC) in Tripoli. The preparation of both bacterial strains was conducted in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines: M02-A11. Both bacteria were subsequently re-cultured on nutritional Mueller Hinton agar (MHA; Difco, Sparks, USA) for 24 hours at 37°C. Subsequently, two to three bacterial colonies were inoculated into 1 mL of Mueller Hinton broth (MHB; Difco, Sparks, USA) using a sterile cotton swab. The bacterial suspension was vortexed for 10 minutes and then incubated for 24 hours at 37°C. Subsequently, 10 µL of the bacterial suspension was introduced into 10 mL of Mueller-Hinton broth (MHB). The turbidity of the inoculum was diluted to roughly above 10^6 CFU/mL concentrations, employing standard broth microdilution and inoculum quantification techniques.

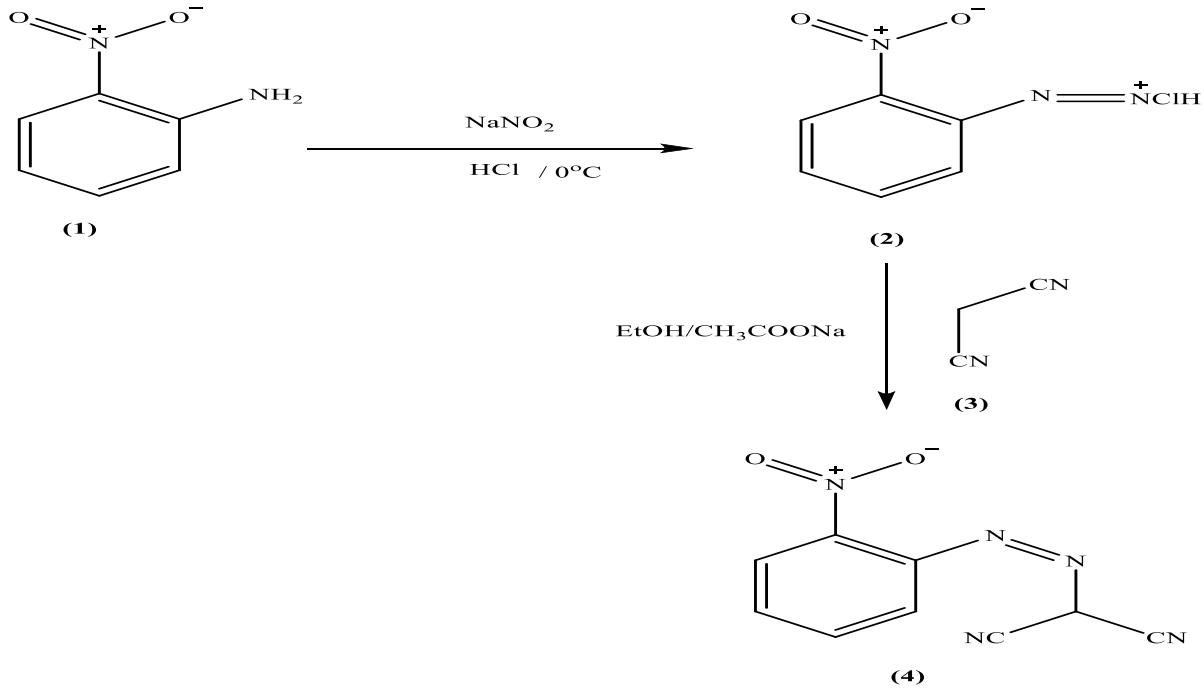
Inoculum quantification was conducted by plating 20 µL of bacterial culture on Mueller-Hinton agar and enumerating the colonies formed after a 24-hour incubation at 37°C.

Antimicrobial Disc Diffusion Test

The antibacterial efficacy of the NPDAM compound was assessed utilizing the disc diffusion method [25]. The bacterial inoculum was uniformly distributed across the surface of Mueller-Hinton agar (MHA) plates with a sterile cotton swab. Sterile 6 mm Whatman filter paper discs (Germany) were saturated with 10 µL of the NPDAM solution at concentrations of 10, 5, and 1 mg/mL. The discs were meticulously positioned on the contaminated agar plates, with sufficient distance between them. Positive controls consisting of 10 µg of Streptomycin for Gram-negative bacteria and 10 µg of Vancomycin for Gram-positive bacteria, together with a negative control of 10% DMSO, were also applied to the plates. After incubation at 37°C for 24 hours, the inhibitory zones were recorded and measured in millimeters.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Values

The Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) were ascertained in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations [25]. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of NPDAM at concentrations of 1, 5, and 10 mg/mL against MRSA and E. coli were evaluated on a 96-well microtiter plate utilizing the normal two-fold serial microdilution technique, with a bacterial inoculum of about 10^6 CFU/mL. One hundred microliters of each concentration of the NPDAM compound were combined and serially diluted two-fold with the test microorganisms in Mueller-Hinton broth (100 µL). Column 12 of the microtiter plate had the highest concentration of the chemical, whilst column 3 contained the lowest concentration. Column 2 functioned as the positive growth control (MHB with inoculum only), while column 1 acted as the negative control (MHB alone, devoid of inoculum or antibacterial agent). The microtiter plate was thereafter incubated aerobically at 37°C for 24 hours. The MIC was established as the minimal concentration of the antibacterial agent that entirely suppressed observable bacterial growth. The minimum bactericidal concentration (MBC) values for each bacterial species were established by discarding the media from wells exhibiting no apparent growth, thereafter, subculturing into Mueller-Hinton agar (MHA) plates. The plates were then incubated at 37°C for 24 hours until noticeable growth was observed in the control plates. The MBC was determined as the minimal concentration sufficient to entirely eradicate the bacteria [26].



Scheme 1

Statistical Analyses were conducted using Windows Excel 2010, with results presented as mean ± SD of three replicates.

Results and discussion

The outcomes of the antibacterial disc diffusion assay for NPDAM against MRSA and E. coli are displayed in Table 1 and Figures 1 and 2. The data demonstrate that NPDAM shown significant antibacterial activity against MRSA at doses of 1, 5, and 10 mg/mL, resulting in inhibition zones of 29.6, 32.6, and 33.0 mm, respectively. Conversely, the identical concentrations exhibited relatively less inhibitory efficacy against E. coli, producing inhibition zones of 10.0, 11.6, and 14.6 mm, respectively. The typical positive controls generated inhibition zones of between 16 and 21 mm for MRSA and E. coli.

Table 1: Disc diffusion of NPDAM against tested bacteria

Concentration of synthesized compound	MRSA (SA121) clear zone(mm) ± SD	*E. coli* (EC49) clear zone(mm) ± SD
1 mg/mL	29.6±0.57	10.0±0.00
5 mg/mL	32.6±1.52	11.6±1.15
10 mg/ mL	33.0±0.57	14.6±0.57
Control +	16.0±0.35	21.0±0.00
Control -	NA	NA

Positive control; Vancomycin for MRSA and Streptomycin for *E. coli*, Negative control; DMSO, NA: No Activity, Results were expressed as means ± sd. Significant differences in means (triplicate).

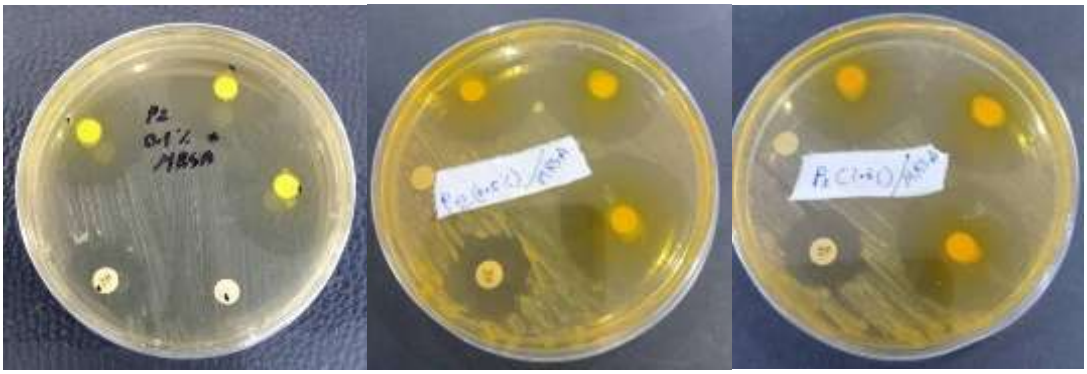


Figure 1: Disk diffusion assay of NPDAM at several doses, with Vancomycin as the positive control and 10% DMSO as the negative control against MRSA.

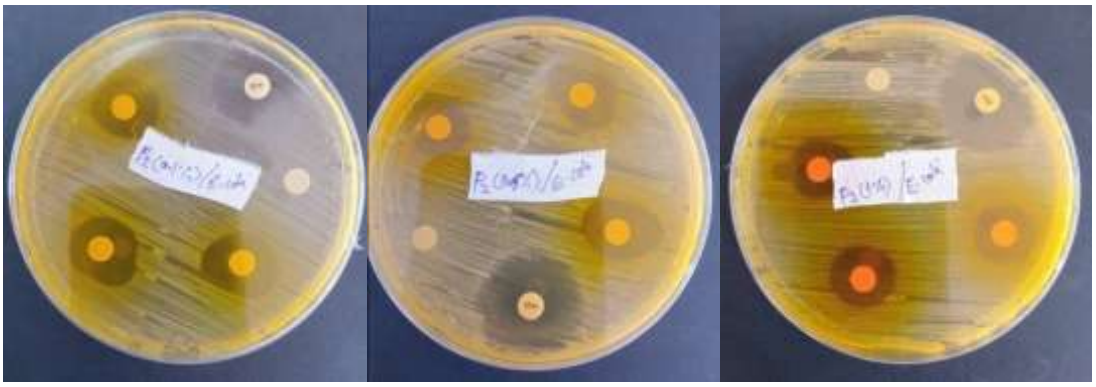


Fig 2: Disk diffusion assay of NPDAM at various concentrations, with Streptomycin as the positive control and DMSO 10% as the negative control against E. coli.

The susceptibility of MRSA and E. coli to NPDAM at various concentrations was evaluated based on their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. The MIC and MBC values are provided in Table 2 and Figures 3 and 4.

Table 2: Minimum inhibitory concentrations (MICs) and Minimum bactericidal concentration (MBCs) of NPDAM

Concentration of NPDAM	MRSA (SA121)		*E. coli* (EC49)	
	MIC	MBC	MIC	MBC
1 mg/mL	19.5	39.0	39.0	78.1
5 mg/mL	4.89	9.78	9.78	19.56
10 mg/mL	0.97	1.95	0.97	1.95

MIC and MBC values are expressed in mg./ mL

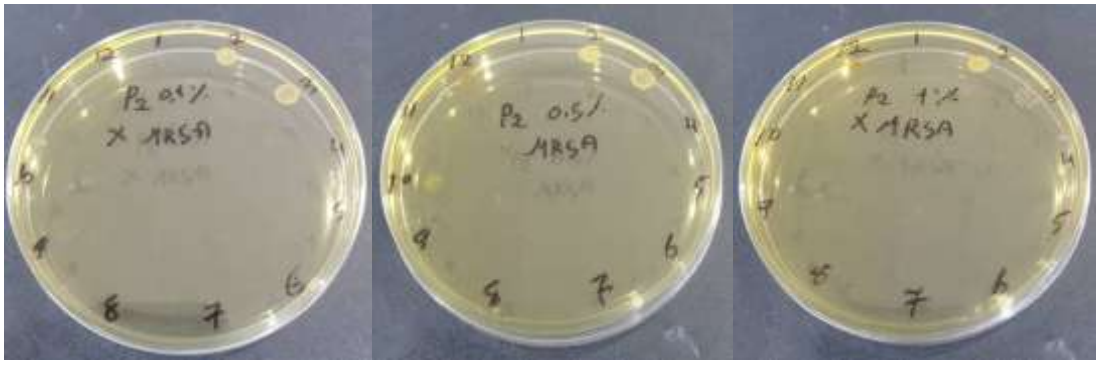


Fig 3: MIC and MBC values of NPDAM at different concentrations against *MRSA*.

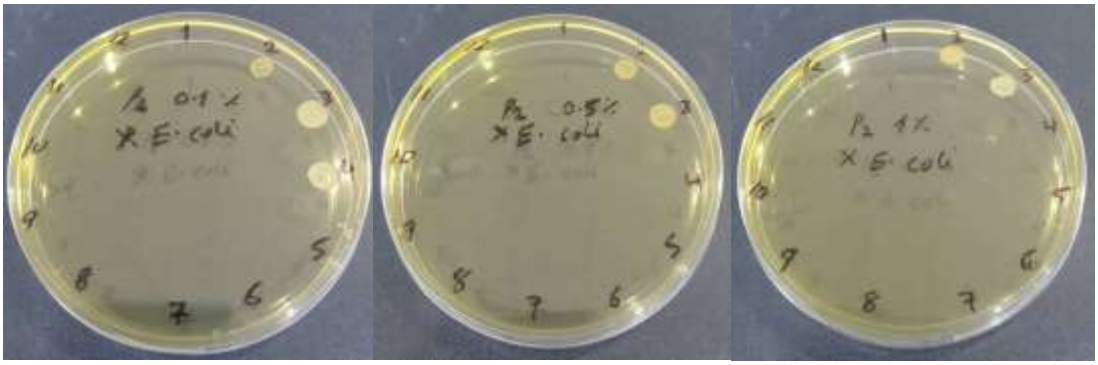


Fig 4: MIC and MBC values of NPDAM at different concentrations against *E. coli*.

Both bacteria demonstrated susceptibility to NPDAM. The low MIC values were recorded at concentrations of 1 and 5 mg/mL (19.5 and 4.89 µg/mL, respectively) against MRSA, in contrast to E. coli, which exhibited inhibition at 39.0 and 9.78 µg/mL, respectively, at the same concentrations. The lowest MIC values (0.97 µg/mL) were observed against both bacteria at a concentration of 10 mg/mL. The MBC values varied from 39.0 to 1.95 µg/mL against MRSA, while exhibiting slightly reduced bioactivity, with MBC values ranging from 78.1 to 1.95 µg/mL against E. coli.

The data indicate that this chemical significantly impacted both bacterial species. It is significant that its effect was more pronounced on MRSA (SA121), a Gram-positive bacterium, than on E. coli (EC49), which exemplifies Gram-negative microorganisms. The disparity in the compound's effect on the two bacterial kinds may stem from its distinct influence on their cell walls, given the structural differences between them. This molecule may affect specific internal cellular components or hinder essential biological processes in bacteria, thereby impeding their growth, depending on the bacterial type.

Conclusion

In conclusion, NPDAM had an effect at all concentrations on the two bacterial strains utilized in this study. It is noteworthy that it demonstrated a more pronounced effect on MRSA (SA121) than on E. coli (EC49). Consequently, it is advisable to utilize this chemical as a synthetic antibacterial agent following the completion of the suggested toxicity assessments to confirm the absence of deleterious side effects during its application. It is recommended to concentrate on its application against Gram-positive bacteria.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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