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EVALUATION OF THE BIOLOGICAL PROPERTIES OF RDX SYNTHESIZED *VIA* EFFICIENT NITROLYSIS OF HEXAMINE BY HETEROPOLYACIDS USING ANALYSIS OF INFLUENTIAL PARAMETERS OBTAINED FROM CENTRAL COMPOSITE DESIGN METHOD

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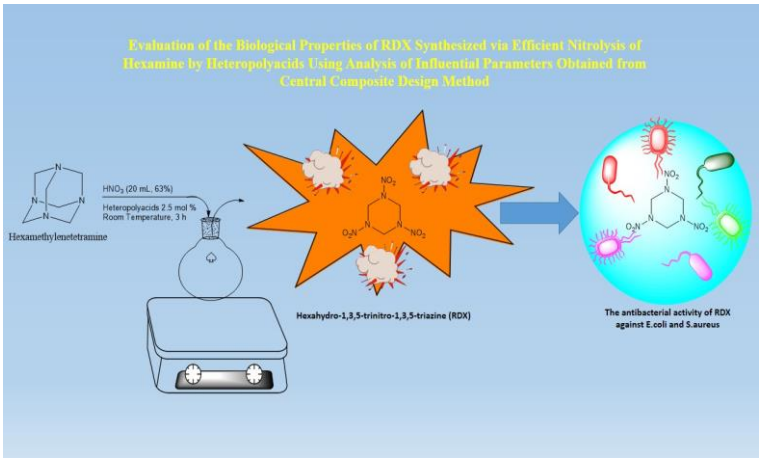
Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX or hexogen among other names), is a typical single compound explosive and one of the most important explosives with the formula $C_3H_6N_6O_6$. A new efficient method of synthesizing Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) was introduced using nitrolysis of hexamethylenetetramine (Hexamine) with HNO_3 in the presence of heteropolyacids at room temperature. Benefits include high product yield, short reaction time, mild conditions and eco-friendliness. RDX's antibacterial properties against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) were evaluated using agar well diffusion at different pH levels and RDX amounts. Statistical methods like Response Surface Methodology and Central Composite Design were used for optimization. Results showed RDX inhibited *Escherichia coli* and *Staphylococcus aureus* growth, with a stronger effect on E. coli. RDX concentration and pH levels affected *Escherichia coli* inhibition zone size but not for *Staphylococcus aureus*.



INTRODUCTION

Nitrolysis is a fundamental reaction in synthetic chemistry that has attracted considerable attention from researchers and is extensively applied in industrial settings.[1-3] One notable application is the synthesis of high-energy materials, such as hexahydro-1,3,5-trinitro-1,3,5-triazacyclohexane.

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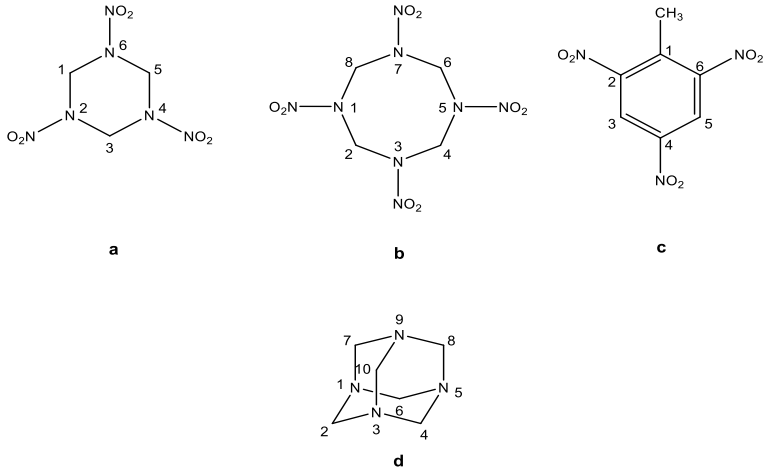
Hexahydro-1,3,5-trinitro-1,3,5-triazine (Scheme 1a), commonly known as RDX or hexogen, is a prominent single-component explosive, recognized for its significance with the chemical formula $C_3H_6N_6O_6$.[4,5] This compound is a white, odorless, and tasteless solid frequently utilized as a military explosive due to its high energy density, rapid reaction rate, and low sensitivity to external factors. It is categorized chemically as a nitroamine, alongside HMX (Scheme 1b), which is considered more energetic than TNT (Scheme 1c). RDX was widely deployed during World War II and continues to be prevalent in military applications. Since its initial synthesis by Henning in 1899,[6] numerous methods for producing RDX have been developed. These methods typically involve the direct nitrolysis of hexamethylenetetramine (HA) using concentrated nitric acid (HNO_3) (known as the Woolwich process), or a mixture of concentrated nitric acid with ammonium nitrate (NH_4NO_3) in acetic anhydride (referred to as the Bachmann process), along with various catalysts and reagents such as perfluorooctanesulfonic acid (PFOS) ($CF_3(CF_2)_7SO_3H$) and Brønsted acidic ionic liquids.[7–15]

Heteropolyacids (HPAs) are effective catalysts that facilitate a broad spectrum of reactions in both homogeneous and heterogeneous systems, including liquid-solid, gas-solid, and liquid-liquid biphasic environments. They provide more efficient and environmentally sustainable alternatives to conventional mineral acids.[16,17] Acting as Brønsted acids, HPAs offer various advantages over liquid acid catalysts. The solid form of HPAs has gained considerable interest in the field of organic synthesis due to their straightforward work-up processes, simple filtration, and potential for cost savings and waste reduction through catalyst recycling and reuse. These acids exhibit significantly enhanced catalytic activity compared to traditional catalysts, such as mineral acids, mixed oxides, and zeolites. In organic settings, the molar catalytic activity of HPAs can be 100 to 1000 times greater than that of sulfuric acid (H_2SO_4), allowing for reactions to be conducted at lower concentrations of catalysts and/or at reduced temperatures. Additionally, HPAs are stable, relatively non-toxic crystalline materials, making them a preferred choice due to their safety and ease of handling.[18] Keggin-type HPAs, generally represented by the formula $[XM_{12}O_{40}]_n^-$, where X is the heteroatom (commonly phosphorus (P^{+5}) or silicon (Si^{+4})) and M is the addenda atom (typically tungsten (W^{+6}) or molybdenum (Mo^{+6})), are vital in catalysis. These compounds are extensively employed as acids and oxidation catalysts in organic synthesis and have a variety of industrial uses. Among the Keggin-type HPAs, those substituted with transition metals, such as $H_3[PMo_{12}O_{40}]$, have been identified as effective catalysts in various organic reactions.[19]

Bacterial infections are now recognized as one of the primary causes of mortality globally. Antibiotics have become widely used due to their effectiveness, affordability, and capacity to treat these infections, positioning them as the go-to option for such ailments. However, the emergence of antibiotic resistance has turned into a significant global challenge. Research indicates that the increasing use of antibiotics among patients correlates with heightened bacterial resistance within their systems. This situation has driven scientists to seek viable alternatives.[20]

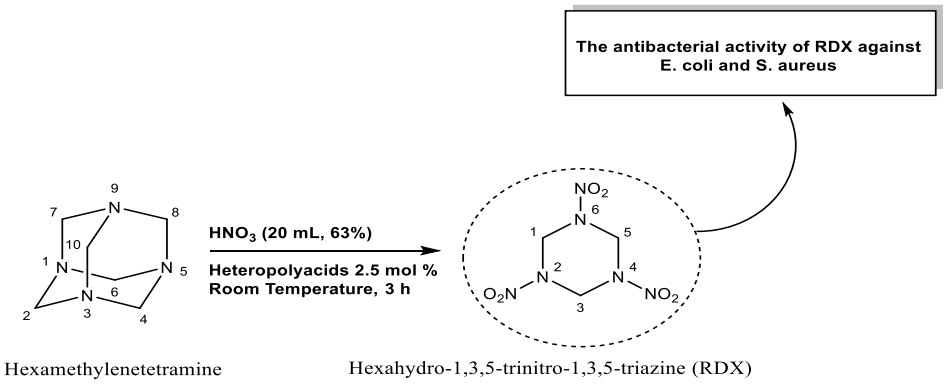
Response Surface Methodology (RSM) encompasses a set of statistical and mathematical tools that are valuable for modeling and analyzing situations where multiple variables impact a particular response of interest.[21] A key goal of RSM is to optimize response variables. The traditional one-factor-at-a-time approach to optimizing several parameters can be quite time-intensive; thus, RSM offers a more efficient alternative, particularly when interactions between factors play a crucial role.[22,23]

Given the explanations provided above, it appears that pursuing a viable method to attain high-yield RDX could be appealing. To the best of our knowledge, scientific sources have not previously reported on the nitrolysis of hexamethylenetetramine (Scheme 1d) to synthesize RDX as an energetic explosive in the presence of some heteropolyacids and its antibacterial properties.



Scheme 1 – (a) Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX); (b) octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX); (c) 2,4,6-trinitroluene (TNT); (d) hexamethylenetetramine.

Therefore, as part of our continuous research efforts in designing and developing the synthesis of organic compounds,[24–31] this paper presents a simple and efficient method for synthesizing RDX as an energetic explosive through the nitrolysis of hexamethylenetetramine (Scheme 2). This synthesis was accomplished in the presence of heteropolyacids, resulting in an excellent yield of 87%. Furthermore, this study aimed to evaluate the antibacterial activity of RDX against *Escherichia coli* and *Staphylococcus aureus* using Response Surface Methodology.



Scheme 2 – Synthesis of RDX through nitrolysis of hexamethylenetetramine in the presence of heteropolyacids and its antibacterial properties investigation.

RESULTS AND DISCUSSION

In order to study of antimicrobial activity of RDX, the RSM with central composite design (CCD) was applied containing two factors. The factors include the concentration of RDX and pH values, which were selected as independent variables. Additionally, the inhibition zone diameters of E. coli and S. aureus were chosen as the response variables. The Minitab statistical software (version 20.04) was used for experimental design and data analysis.[32] The levels of independent variables are shown in Table 1. A total of 13 experiments designated using the Minitab software and statistical analysis was performed to evaluate the analysis of variance (ANOVA). It should be noted that 95% level of confidence was used in this research. In this study, the antimicrobial activities of RDX were investigated by growing S.aureus and E. coli colonies on MHA plates, supplemented with different concentrations of RDX at different pH values which designed by CCD. According to the designed experiments, 13 experiments were carried out and the results were shown in Table 2.

The regression model for inhibition zone diameter of RDX for *E. coli* as response variables was found in the linear regression equation as follows:

$$E.coli = 11.1 + 3.13\ RDX + 3.42\ pH \quad (1)$$

The ANOVA results for the model are shown in Table 3 in the supplementary information file. The P-values of RDX and pH are less than 0.05 and consequently it can be concluded that RDX and pH are significant. Additionally, the F-value of 9.9 and P-value < 0.05 indicate that the response surface linear model is significant. Table 4 in the supplementary information file shows the test of significance for regression coefficients of Eq. (1). The P-vales of this table also confirm that all terms of model are significant (P-value < 0.05). It is noteworthy that the positive sign of RDX and pH coefficients indicate that the inhibition zone diameters tend to increase when RDX and pH increase.

Table 1

Levels of two independent variables used for designing of central composition design

	Levels				
Factors	–1.41	–1	0	1	1.41
RDX (mg/L)	29.29	50	100	150	170.71
pH	4.17	5	7	9	9.83

Table 2

Central composite design and responses for *E. coli* and *S. aureus*

Run number	RDX [a] (mg/L)	pH [b]	E. coli [c] (mm)	S. aureus [d] (mm)
1	50	9	17.3	16.0
2	100	7	10.9	6.3
3	100	7	10.8	7.7
4	100	7	8.4	6.6
5	100	7	9.5	5.4
6	150	9	16.5	17.2
7	100	9.83	14.7	14.0
8	50	5	6.2	9.3
9	29.29	7	2.5	1.0
10	100	4.17	8.5	7.6
11	150	5	9.0	8.7
12	100	7	11.2	9.7
13	170.71	7	18.8	18.0

a: hexahydro-1,3,5-trinitro-1,3,5-triazine
b: In chemistry, pH, also referred to as acidity or basicity, historically denotes “potential of hydrogen”.
c: Escherichia coli
d: Staphylococcus aureus

Figure 1 shows the scatter plots of E. coli, versus the factors RDX and pH. It can be easily seen from the figure that inhibition zone diameter tends to increase when RDX and pH increase. These findings are in complete agreement with the previous results obtained from the linear regression model. The regression model for inhibition zone diameter of RDX for S.aureus as response variables was found in the linear regression equation as follows:

$$S.aureus = 7.14 + 3.08RDX + 3.03pH + 1.84RDX \times RDX + 2.49pH \times pH + 0.45RDX \times pH \quad (2)$$

The ANOVA results for the model are shown in Table 3. The P-values of RDX and pH are less than 0.05 and consequently it can be concluded that RDX and pH are significant. Additionally, the F-value of 9.9 and P-value < 0.05 indicate that the response surface linear model is significant. Table 4 shows the test of significance for regression coefficients of Eq. (1). The P-vales of this table also confirm that all terms of model are significant (P-value < 0.05). It is noteworthy that the positive sign of RDX and pH coefficients indicate that the inhibition zone diameters tend to increase when RDX and pH increase. It can be concluded from the P-values of regression coefficients that none of model terms are significant. In other words, the factors RDX and pH have no effect on inhibition zone diameter

Table 3

Regression analysis by ANOVA for *E. coli*

Source [a]	DF [b]	Adj SS [c]	Adj MS [d]	F-Value [e]	P-Value [f]
Model	2	172.075	86.038	9.90	0.004
Linear	2	172.075	86.038	9.90	0.004
RDX	1	78.448	78.448	9.03	0.013
pH	1	93.627	93.627	10.77	0.008
Error	10	86.905	8.690		
Lack-of-Fit	6	81.333	13.555	9.73	0.023
Pure Error	4	5.572	1.393		
Total	12	258.980			

a: The source of the variation in the data.
b: The degrees of freedom in the source.
c: Adjusted sums of squares.
d: Adjusted mean squares.
e: The F-statistic value
f: Probability value

Table 4

Testing of the significance of the regression coefficients for model

Term	Coef [a]	SE Coef [b]	T-Value [c]	P-Value
Constant	11.100	0.818	13.58	0.000
RDX	3.13	1.04	3.00	0.013
pH	3.42	1.04	3.28	0.008

a: Coefficients
b: Standard errors of the coefficients
c: t-statistics

Table 5

Regression analysis by ANOVA for *S. aureus*

Source [a]	DF [b]	Adj SS [c]	Adj MS [d]	F-Value [e]	P-Value [f]
Model	5	209.737	41.9474	2.99	0.093
Linear	2	149.415	74.7075	5.32	0.039
RDX	1	75.901	75.9012	5.40	0.053
pH	1	73.514	73.5137	5.23	0.056
Square	2	59.512	29.7561	2.12	0.191
RDX×RDX	1	23.616	23.6160	1.68	0.236
pH×pH	1	43.218	43.2178	3.08	0.123
2-Way Interaction	1	0.810	0.8100	0.06	0.817
RD×pH	1	0.810	0.8100	0.06	0.817
Error	7	98.352	14.0503		
Lack-of-Fit	3	87.460	29.1534	10.71	0.022
Pure Error	4	10.892	2.7230		
Total	12	308.089			

a: The source of the variation in the data.
b: The degrees of freedom in the source.
c: Adjusted sums of squares.
d: Adjusted mean squares.
e: The F-statistic value
f: Probability value

The quality of fitted models was evaluated based on the adjusted coefficient of determination denoted by R2adj. It is well known that $0 \leq$ R2adj.≤ 1 and values close to 1 imply that the associated regression model can be used as an appropriate predictor of response variable. The R2adj for Eqns. (1) and (2) was 0.6644 and 0.6808, respectively. These values of R2adj are relatively high indicating that the proposed regression models have ability to predict inhibition zone

diameter of silver nanoparticle for *E. coli* and *S. aureus* with the help of two factors. The F-values of models were significant (P-value < 0.05), which confirmed the suitability of the proposed models. In addition, the P-values showed that all model terms in Eq. (1) was significant (P-value < 0.05). The linear regression models proposed in Eqns. (1) and (2) are very informative.

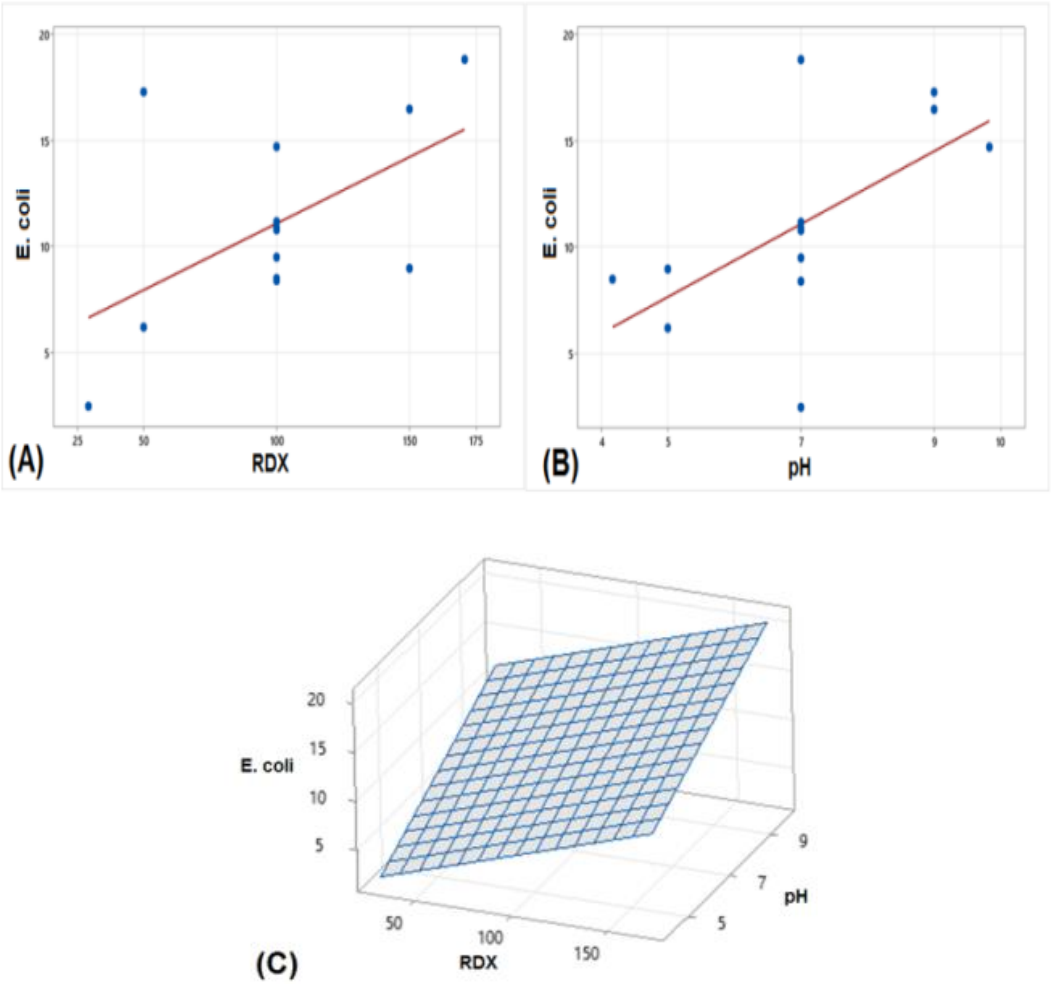


Fig. 1 – (a) Scatterplot of *E. coli versus* RDX, (b) Scatterplot of *E. coli versus* pH; (c) Surface Plot of *E. coli versus* RDX and pH.

EXPERIMENTAL

Apparatus, materials, and measurements

All chemicals were purchased from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland), and used without further purification. Commercially available solvents and reagents were used without further purification. Hexamethylenetetramine was purchased from Merck (Darmstadt, Germany) (CAS Number: 100-97-0). Hydrate of $H_3PW_{12}O_{40}$ was purchased from Fluka chemical Co. Hydrates of $H_3PMo_{12}O_{40}$ and $H_7SiW_9V_3O_{40}$ was prepared according to a published procedure.[18] Before use, the catalyst was dried at 50 °C. Melting points were determined on an Electrothermal 9100 apparatus. The Fourier transform infrared spectroscopy (FT-IR) spectra were measured on a Jasco 6300 FT-IR spectrometer (KBr disks). ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker 250 MHz instrument model WG-300 and the δ units were referenced to tetramethylsilane internal standard. The antibacterial activity was studied using the agar well diffusion method against *E. coli* and *S. aureus.*

Procedure for preparation of RDX with the nitrolysis of hexamethylenetetramine in the presence of heteropolyacids

In a round-bottomed glass flask, nitric acid 63% (20 mL) was gradually added to a mixture of hexamethylenetetramine (HA) (2 mmol, 280 mg) and heteropolyacid (2.5 mol % with respect to HA) over 15 minutes. Then, the reaction mixture was

stirred for 3 h at room temperature. Then the mixture was filtered and put in a cool place (10 °C). Completion of the reaction was accompanied by the formation of crystals. Filtration was done and the obtained solid was washed with distilled cool water, and then dried. The melting point of the synthesized product was 204–205 °C. Under these conditions, the reaction yield was 87%. The synthesized product is a known compound. Its structure was confirmed using various spectral techniques like IR, ^{1}H, ^{13}C-NMR, and some of its physical properties and compared with data reported in the literature.[13]

Spectroscopic data for Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)

^{1}H-NMR (250 MHz, DMSO-d_6, ppm): δ_H= 5.99 (s, 6H, $3CH_2$)

Evaluation of antibacterial effects of RDX

The antibacterial activities of RDX were tested on two bacteria obtained from clinical specimens: *Escherichia coli* (*E. coli*), a Gram-negative bacterium, and *Staphylococcus aureus* (*S. aureus*), a Gram-positive bacterium. These strains were maintained on Nutrient Agar at 4°C for further studies. Antibacterial activity of RDX against *E. coli* and *S. aureus* was studied using the agar well diffusion method. The petri dishes including semi solid Mueller Hinton agar (MHA) were cultured with 1.5 × 108 CFU/mL suspensions of test bacteria. Different concentrations of RDX with different pH values of the medium culture (the values designed by central composite design) were inoculated into the wells (6 mm diameter). The plates were incubated for 24 hours at 37°C. Antimicrobial activity was measured based on the diameter of inhibition zone in mm. The pH adjusted by addition of 1 M NaOH and 1 M HCl.[20]

CONCLUSION

In conclusion, we have developed a practical, simple and efficient reaction for the synthesis of Cyclotrimethylenetrinitramine (RDX). The method has several advantages, including high yields of product, short reaction time, easy experimental work-up, and Eco friendly. In this study, the evaluation of the antibacterial activity of the RDX was performed using Response Surface Methodology against *E. coli* and *S. aureus*. The results of this study revealed that RDX showed the antibacterial effect on *E. coli* and *S. aureus*. The antibacterial activity of RDX on *E. coli* was more than that of *S. aureus*. In addition, the results showed that the RDX concentrations and pH values were effective on the inhibition zone diameter of *E. coli*, while these parameters had no effect on the inhibition zone diameter of *S. aureus*. The results also showed that, as the amount of RDX concentration and pH values increased the diameter of the inhibition zone increased for *E. coli*. Another result of this study was that the RDX concentration and pH values did not show any interaction. This means that in this experiment, pH had no effect on the antibacterial effect of RDX. The results of this research showed that although RDX had an antibacterial effect, changing the RDX concentration and the pH values had no effect on the inhibition zone diameter. Although the results showed that this new chemical has an antimicrobial effect on some bacteria such as *E. coli* and *S. aureus*, more research is still needed. In particular, its effects on the human body are used as an antimicrobial drug.

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