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ORIGINAL RESEARCH ARTICLE

Using green chemistry (deep eutectic solvents) to synthesize pyrazol-4-yl-thiazolidin-4-one derivatives and evaluate their antibacterial activity

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ABSTRACT

This study employed a green chemistry approach utilizing two deep eutectic solvents (DESs), namely Reline and Malonine, as environmentally friendly ionic liquids (ILs). These solvents were readily prepared from choline chloride combined with either two moles of urea or one mole of malonic acid, respectively. Acting as dual-function catalysts, they are characterized by their low melting points and suitability as attractive media for organic reactions, in addition to being cost-effective, non-toxic, bio-renewable, and biodegradable.

The two DESs were applied to improve the yields of Schiff bases, specifically 1,5-dimethyl-4-(substituted styryl)-2-phenyl-1,2-dihydro-3H-pyrazol-3-one, I(a–c). The yields increased to 95% in methods B and C, compared with 76–80% obtained using the conventional method (A). Moreover, reaction times were reduced from 120 minutes in method A to just 30 minutes in methods B and C.

Similarly, in methods E and F, solvent-free conditions combined with simplified procedures led to high yields (95%), compared with only 50–51% achieved using the conventional method (D). Reaction times were also reduced to one quarter of those in method D during the synthesis of the novel compounds II(a–c).

Both DESs (Reline and Malonine) could be efficiently recovered and reused up to seven times, after which they were reactivated by heating with choline chloride.

The antibacterial activities of the synthesized compounds II(a–c) were evaluated using standard in vitro assays against four pathogenic microorganisms: *Bacillus cereus* and *Staphylococcus aureus* (Gram-positive), as well as *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative). Among them, *Staphylococcus aureus* exhibited the highest inhibition with Compound a (31 mm/mg), outperforming penicillin (16 mm/mg and 30 mm/mg, respectively). Compound b showed the strongest activity against *Pseudomonas aeruginosa* (40 mm/mg), significantly higher than penicillin (24 mm/mg). While penicillin demonstrated the strongest inhibition against

Bacillus cereus (24 mm/mg), Compound c exhibited a comparable effect (22 mm/mg).

Overall, these findings highlight the promising antibacterial potential of Compounds b and c, with some surpassing penicillin against certain bacterial strains. Further investigations into their mechanisms of action and toxicity are recommended to assess their potential as novel antibacterial agents.

Keywords: green chemistry; deep eutectic solvents; pyrazol-4-yl-thiazolidin-4-one derivatives; antibacterial evaluation

1. Introduction

One important class in organic synthesis is the derivatives containing the functional group known as imine groups, commonly referred to as Schiff bases. Their significance lies in their diverse biological activities, including anti-tumor, antiviral, antifungal, and antibacterial properties[1–3].

Traditionally, Schiff bases are synthesized through the nucleophilic attack of an appropriate amine on the electrophilic carbonyl carbon of an aldehyde or ketone via a tetrahedral mechanism. Catalysts, such as Lowry-Bronsted acids—most commonly acetic acid—facilitate the reaction by protonating the carbonyl oxygen or aiding in the elimination of a water molecule, as shown in (**Figure 1**)[4].

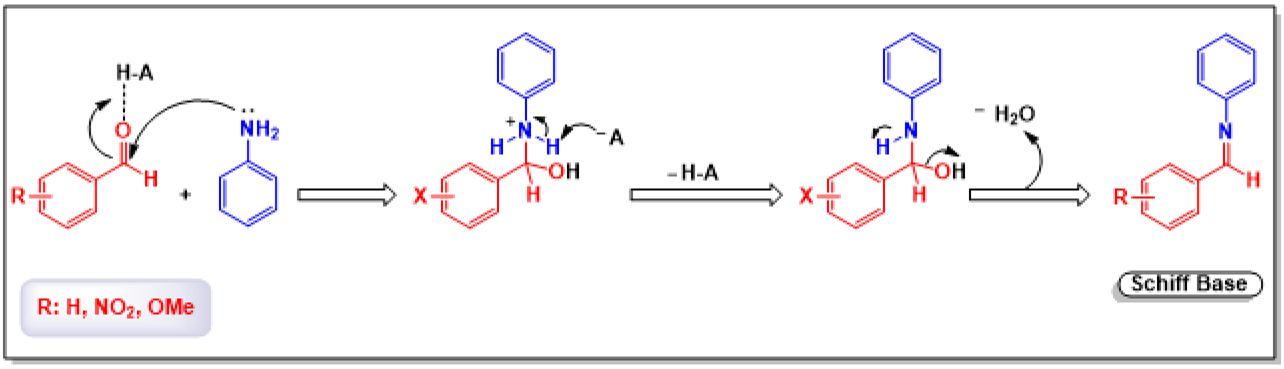

Figure 1. Role of lewis acid catalysts in schiff base formation.

In this study, a comparative investigation was carried out to synthesize Schiff bases incorporating the pharmacologically significant pyrazol-5-one moiety, which is known to exhibit a wide range of biological activities, including antibacterial, anti-inflammatory, antihypertensive, antifungal, anti-HIV, antitumor, and anticonvulsant effects[5–7]. Notably, pyrazol-3-one forms the core structure of 4-aminophenazone, a well-established nucleus widely used as an analgesic for acute migraines, as well as for its antipyretic and anti-inflammatory properties[8,9]. In this work, the pyrazol-3-one moiety was expected to enhance biological activity when conjugated with thiazolidin-4-one to produce the target compounds[10,11]. This is attributed to the presence of the pyrazol-3-one moiety linked with thiazolidin-4-one, a five-membered heterocyclic ring containing nitrogen and sulfur atoms along with a carbonyl group at the 4-position, which contributes to its role as a novel antibacterial pharmacophore[12,13]. Furthermore, thiazolidin-4-one derivatives are recognized as important pharmaceutical scaffolds due to their broad-spectrum antibacterial activity. Their diverse biological activities have motivated pharmaceutical chemists to design and synthesize new derivatives with promising therapeutic potential[14,15].

The comparative synthesis of Schiff bases I(a–c), which served as synthons for the preparation of the final target derivatives II(a–c), was performed using both the conventional method with glacial acetic acid (GAA) and alternative green methods employing deep eutectic solvents (DESs) such as Reline (choline chloride–urea) and Malonine (choline chloride–malonic acid)[16]. Subsequently, these Schiff bases were reacted directly with an excess of thioglycolic acid (mercaptoacetic acid) under reflux in dioxane, leading to the one-pot cyclization and formation of the target thiazolidin-4-one derivatives II(a–c). This cyclization reaction proceeds via the nucleophilic attack of the imine nitrogen atom on the carbonyl carbon of mercaptoacetic acid, resulting in the construction of the thiazolidin-4-one ring system[17,18]. The proposed nucleophilic attack mechanism was previously suggested by Hoan five years ago and is illustrated in **Figure 2**[19].

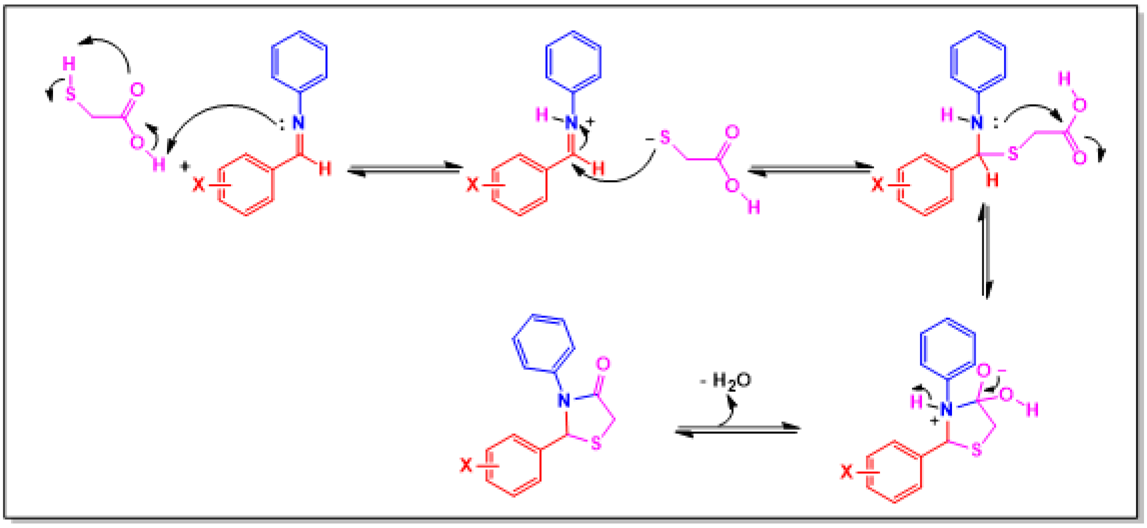

Figure 2. Proposed Nucleophilic Mechanism for the Formation of Thiazolidin-4-one Ring.

Deep eutectic solvents (DESs), considered promising alternatives to ionic liquids (ILs), offer several advantages in organic synthesis. They are eco-friendly (green chemistry), inexpensive, and function dually as both solvents and catalysts. Moreover, they enable convenient procedures, rapid or even one-pot reactions, and excellent product yields[20]. DESs are simple to prepare, even in a test tube without the need for conventional organic reaction systems, by mixing hydrogen bond donors (HBDs) such as urea or malonic acid (used in this work as Reline and Malonine, respectively) with a quaternary ammonium salt such as choline chloride (ChCl), which is safe, biodegradable, and of low toxicity[21].

It is noteworthy that hydrogen bond donors like urea or malonic acid reduce the lattice energy of the mixture components, thereby lowering their freezing points. As a result, Reline melts at 12°C, whereas Malonine melts at 60°C[16]. Despite extensive efforts in synthesizing thiazolidin-4-one derivatives, there remains a need to prepare a wide range of analogues based on pharmacologically active scaffolds such as 4-aminophenazone, i.e., the 1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazolyl moiety (DOPDP). This nucleus has been widely studied for its analgesic, anti-inflammatory, and antipyretic properties, providing a basis for exploring additional biological activities[22,23].

In addition to enhancing yields and shortening reaction processes, the use of eco-friendly DESs (Reline and Malonine) aligns with the principles of green chemistry, which are emphasized in this laboratory. These considerations inspired the design and synthesis of three novel "pyrazol-4-yl-thiazolidin-4-one" derivatives with the general structure illustrated in **Figure 3**, followed by an evaluation of their antibacterial activities[24].

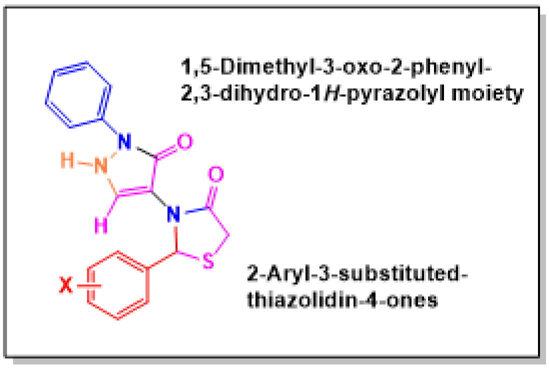

Figure 3. General Structure of Pyrazol-4-yl-Thiazolidin-4-one Derivatives.

In this work, two deep eutectic solvents (DESs), namely Reline and Malonine, were employed as green solvents. Reline was prepared by mixing choline chloride and urea in a 1:2 molar ratio, while Malonine was synthesized from choline chloride and malonic acid in a 1:1 molar ratio. These solvents were subsequently utilized to enhance the production of Schiff bases, specifically 1,5-dimethyl-4-(substituted styryl)-2-phenyl-1,2-dihydro-3H-pyrazol-3-one, I(a–c), and II(a–c). The structures of the newly synthesized compounds were confirmed through their physical properties and further characterized by Fourier-transform infrared (FT-IR) spectroscopy and nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR). The antibacterial activities of compounds II(a–c) were then evaluated in vitro against four pathogenic microorganisms: *Bacillus cereus* and *Staphylococcus aureus* (Gram-positive), as well as *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative).

2. Experimental

All chemicals and solvents were obtained from commercial sources and used without further purification. Deep eutectic solvents (DESs), Reline and Malonine, were prepared according to reported procedures[25], using a 1:2 molar ratio of choline chloride (ChCl) to urea for Reline and a 1:1 molar ratio of ChCl to malonic acid for Malonine. FT-IR spectra (υmax in cm^{-1}) were recorded on a Bruker Alpha FT-IR spectrophotometer (Germany) using the KBr pellet technique. The ^{1}H NMR and ^{13}C NMR spectra of the synthesized compounds were recorded on a Bruker Avance II 400 MHz spectrometer using DMSO-d$_6$ as the solvent and tetramethylsilane (TMS) as the internal reference.

Synthesis of Schiff bases, 1,5-Dimethyl-4-(substituted styryl)-2-phenyl-1,2-dihydro-3H-pyrazol-3-one, I(a–c)

Conventional method using glacial acetic acid (Method A)[9,26]:

A mixture of equimolar amounts (5.8 mmol) of 4-aminophenazone and benzaldehyde (or substituted benzaldehyde), together with a catalytic amount of glacial acetic acid (1 mL), was refluxed in 15 mL of methanol for 120 minutes (Scheme 3). The reaction mixture was cooled and stored overnight in a refrigerator to allow precipitation of the product. The solid obtained was filtered, dried, and recrystallized from a methanol–chloroform mixture (1:1). The physical properties, synthetic details, yields, CHN analysis, FT-IR, and ^{1}H NMR data of the synthesized compounds are presented in **Table 1**.

Green chemistry method using Reline (Method B)[26,27]:

A mixture of equimolar amounts (5.8 mmol) of 4-aminophenazone and benzaldehyde (or substituted benzaldehyde), along with a catalytic amount of Reline (0.5 g), was refluxed in 15 mL of methanol for 30 minutes (Scheme 3). The work-up procedure was identical to that described in Method A. The percentage yields are summarized in **Table 1**.

Green chemistry method using Malonine (Method C)[16]:

A mixture of equimolar amounts (5.8 mmol) of 4-aminophenazone and benzaldehyde (or substituted benzaldehyde), with a catalytic amount of Malonine (0.5 g) in 15 mL of methanol, was refluxed for 30 minutes (**Figure 4**). The reaction mixture was processed as in Method A. The percentage yields are shown in **Table 1**.

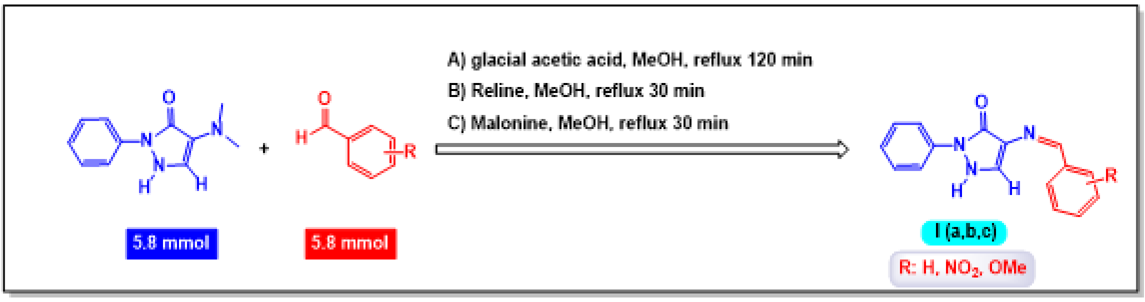

Figure 4. General synthesis of 1,5-Dimethyl-4-(substituted styryl)-2-phenyl-1,2-dihydro-3H-pyrazol-3-one, I(**a**-**c**).

Table 1. Physical and spectral properties of compounds I (**a,b,c**).

Compd. No. I	**X**	**M.p. (˚C) (Color)**	**Yield % Method**			**CHN Analysis Calcd. (found)**	**IR, KBr, ν (cm^{-1})**		**^{1}H NMR (d, ppm) CH=N-**
			A	**B**	**C**		**Imine - C=N-**	**Exocyclic C=O**	
a	H	175-77 (Shiny yellow)	80	>95	>95	C, 74.20(74.18); H, 5.88(6.10); N, 14.42(14.55); O, 5.49(5.50	1586	1485	s. 8.40
b	3-NO_2	217 – 18 (Orange)	76	>95	>95	C, 64.28(64.00); H, 4.79(4.80); N, 16.66(16.55); O, 14.27(14.31)	1600	1480	s, 7.90
c	4-OMe	166-68 (Pale yellow)	80	>95	>95	C, 71.01(70.08); H, 5.96(5.96); N, 13.08(13.28; O, 9.96(9.99)	1617	1480	S, 7.9

Synthesis of Pyrazol-4-yl-thiazolidin-4-one Derivatives II(a–c)

Conventional method (Method D)[28]:

An appropriate 1,5-dimethyl-4-(substituted styryl)-2-phenyl-1,2-dihydro-3H-pyrazol-3-one I(a–c) (1 mmol) was dissolved in 80 mL of toluene, followed by the addition of thioglycolic acid ($HSCH_2CO_2H$, 1 mmol). The reaction mixture was refluxed for 4 hours using a Dean–Stark apparatus (Scheme 4). After cooling, the mixture was washed successively with 3% sodium bicarbonate solution (3 × 10 mL) and brine. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was recrystallized from ethanol. The physical properties, synthetic details, percentage yields, CHN elemental analysis, FT-IR, and ^{1}H NMR data of the synthesized compounds are summarized in **Table 2**.

Green chemistry method using Reline (Method E)[29]:

An appropriate 1,5-dimethyl-4-(substituted styryl)-2-phenyl-1,2-dihydro-3H-pyrazol-3-one I(a–c) (1 mmol) was dissolved in 5 mL of Reline. To this solution, thioglycolic acid ($HSCH_2CO_2H$, 1 mmol) was added (**Figure 5**). The reaction mixture was refluxed for 1 hour using a Dean–Stark apparatus. The subsequent work-up was performed following the same procedure described in Method A. The percentage yields of the obtained products are listed in **Table 2**.

Green chemistry method using Malonine (Method F)[16,30]:

An appropriate 1,5-dimethyl-4-(substituted styryl)-2-phenyl-1,2-dihydro-3H-pyrazol-3-one I(a–c) (1 mmol) was dissolved in 5 mL of Malonine. Thioglycolic acid ($HSCH_2CO_2H$, 1 mmol) was then added to the reaction mixture (Scheme 4). The mixture was refluxed for 1 hour with a Dean–Stark apparatus. The reaction work-up was carried out according to the procedure described in Method A. The percentage yields are presented in **Table 2**.

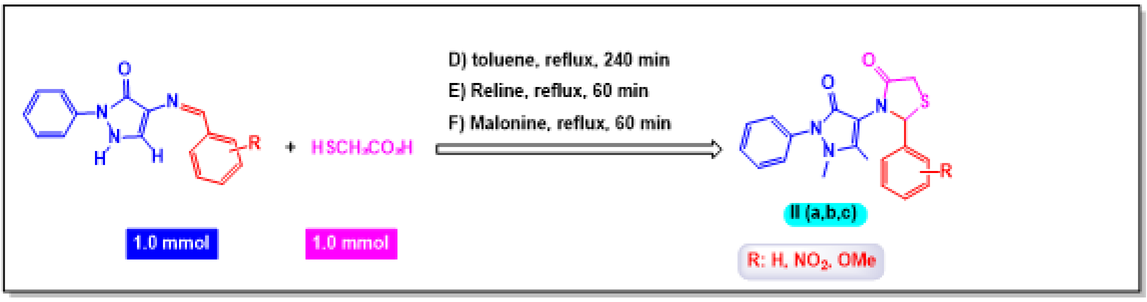

Figure 5. General synthesis of pyrazol-4-yl-thiazolidin-4-one derivatives II (**a-c**).

Table 2. spectral properties of compounds II (**a,b,c**).

Comp. No. II	X	m.p. °C (Color)	Yield % Method			CHN Analysis Calcd. (found)	IR, KBr, ν (cm−1) Thiazolidine Exocylic (C=O)	^{1}H-NMR (d,ppm) Heterocyclic rings protons
			D	E	F			
a	H	133-35 (yellow)	53	96	93	C, 64.08 (64.11); H, 4.48(4.55); N, 12.45(12.50)	1695	9.40(s, NH),6.61(s, H, C2), 3.61-3.77 (q, 2H, C5).
b	3-NO_2	156-58 (white)	50	91	88	C, 56.54(56.60); H, 3.69(4.00); N, 14.65(14.66)	1680	9.33(s, NH),6.75(s, H, C2), 3.55-3.70 (q, 2H, C5).
c	4-OMe	143-45 yellow	51	91	96	C, 64.94(56.00); H, 4.88(5.10); N, 11.96(11.89)	1680	9.40(s, NH),6.58(s, H, C2), 3.55-3.71 (q, 2H, C5).

Deep Eutectic Solvents (Reline and Malonine) Recovery and Reactivate:

After the recrystallization of the products in methods (B), (C), (D), or (F), the mother liquor of aqueous ethanol was evaporated and the remaining Deep eutectic solvents (Reline or Malonine) were dried and used again tell seven times, and the these can be reactivated by heated with a small amount (about the molar weight) of choline chloride that used in above methods experiments, (unpublished procedures). (**Figure 6**) shows the catalyst recovery ratios for the seven cycles.

Figure 6. Number of catalyst recovery.

3. Results and conclusion

Synthesis of Schiff bases I(a–c):

As illustrated in **Figure 6**, Schiff bases—named after their discoverer Hugo Schiff—are also referred to as imines due to the presence of an azomethine linkage (C=N), which is bonded either to a hydrogen or a carbon atom[31]. These compounds were synthesized through the condensation of aromatic aldehydes (unsubstituted benzaldehyde or substituted benzaldehydes containing either electron-donating or electron-withdrawing groups) with 4-aminophenazone (1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazolone), an important pharmacological amine. This approach aimed to demonstrate the influence of substituent effects on the yields of the resulting Schiff bases.

The condensation reaction proceeded smoothly under reflux in an alcoholic solvent, using one milliliter of glacial acetic acid (GAA) as a catalyst for five hours, producing yields below 80% of the desired products[19]. Mechanistically, the reaction involves the nucleophilic addition of the amine to the aldehyde carbonyl group, leading to the formation of a tetrahedral carbinolamine intermediate. This unstable intermediate then undergoes dehydration, losing a water molecule via a base-catalyzed (acetate-mediated) pathway[32].

3.1. Green Chemistry and Deep Eutectic Solvents (DESs):

Deep eutectic solvents (DESs) represent an emerging class of sustainable, low-melting-point ionic liquids with wide applications, particularly as catalytic media in organic synthesis. They are considered environmentally friendly alternatives to traditional organic solvents due to their simplicity of preparation, safety, and low toxicity.

In this work, DESs were employed to facilitate the synthesis of novel organic compounds, particularly heterocycles, while simultaneously increasing product yields. Two types of DESs were prepared: Reline, obtained from mixing choline chloride with urea in a 1:2 molar ratio, and Malonine, prepared from choline chloride and malonic acid. Notably, Malonine was used for the first time in this laboratory[33]. Despite the high melting points of their individual precursors, these eutectic mixtures exhibit remarkably low melting points: 12 °C for Reline[34] and 30–40 °C for Malonine[33].

The advantages of these catalysts in the context of green chemistry are numerous. They are derived from safe, inexpensive, and biodegradable raw materials such as choline chloride, urea, and malonic acid. Furthermore, they generate minimal vapor emissions, thus reducing reliance on harmful organic solvents and mitigating environmental hazards[35,36].

Both DESs—Reline (1 mol choline chloride : 2 mol urea, **Figure 7**) and Malonine (1 mol choline chloride : 2 mol malonic acid)—were prepared through straightforward procedures. They were successfully applied as dual-function catalysts and solvents, leading to significant reductions in reaction times while providing eco-friendly conditions and excellent stoichiometric yields of up to 95%[37]. The proposed mechanism of action of DESs (Reline), which equally applies to Malonine, is presented in **Figure 8**.

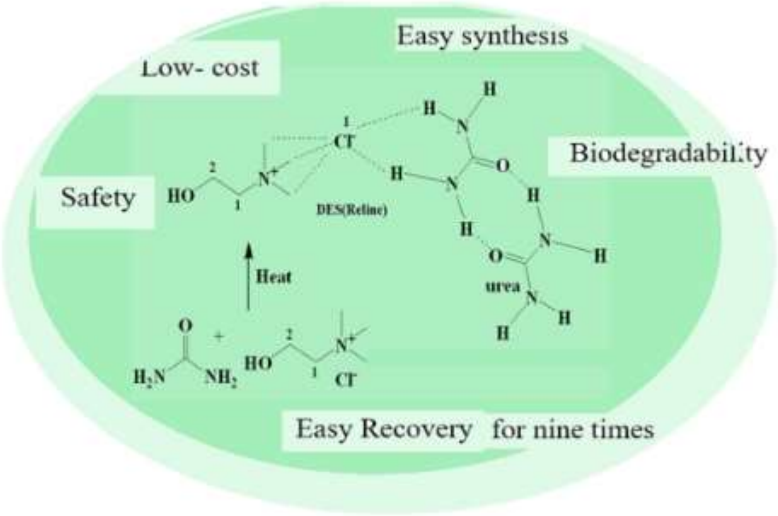

Figure 7. Synthesis of Reline (Choline Chloride with Urea) as a Deep Eutectic Solvent.

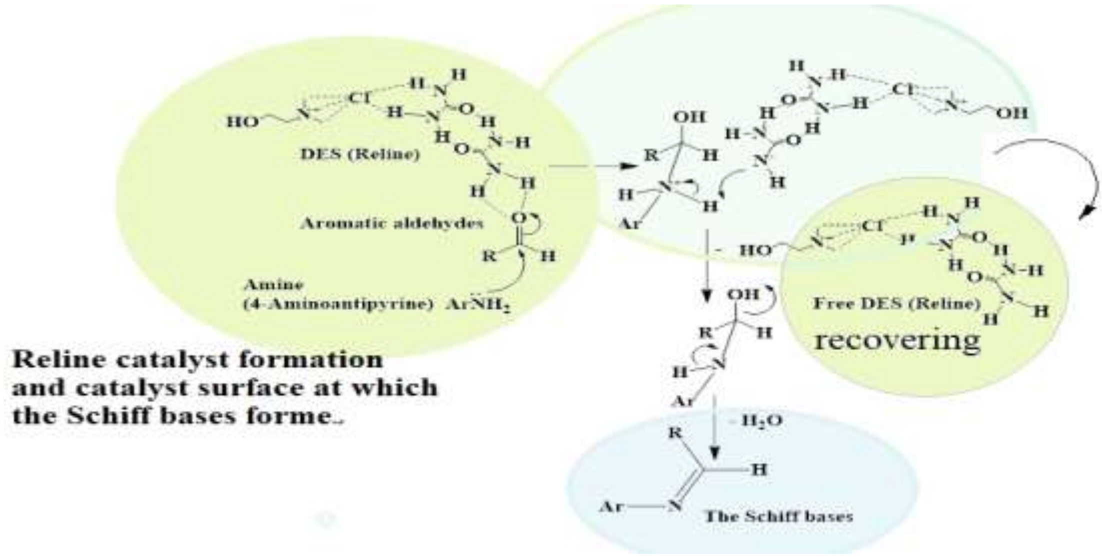

Figure 8. The suggested mechanism of action of DESs Reline.

The Schiff bases chemical structures were deduced from their physical properties in addition to their elemental analysis and studying of their UV, FT-IR, and ^{1}H NMR spectroscopy.

The ^{1}H NMR of these three compounds I (a-c)

4-(Benzylideneamino)-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one, I(**a**):

Methyl protons (-CH_3) at position (5) of the pyrazole ring, in the 2.40 ppm of typically distinct signals for the protons of the allylic system due to their electronic environment while the other methyl protons attach to the pyrazole nitrogen resonates as a doublet around in the 3.20 ppm due to the influence of the electronegative nitrogen. For the two benzene rings of the ten aromatic protons are typically observed in the range of 7.55 – 7.56 ppm the ring attaches to the pyrazole nitrogen ring which produces slight deshielding, while the unsubstituted second benzene ring attaches to the imine bond (-CH=N-C_6H_5) in the 7.35 – 7.52 ppm.

The second compound 4-(o-Nitrobenzylideneamino)-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one, I(**b**), which showed the clear deshielding effects of electrons withdrawing nitro group on the ortho-substituted second benzene ring attaches to the imine bond the (-CH=N-3-NO_2-C_6H_4) in the 7.70- 8.48 ppm, in addition to the imine group which also produce the downfield shifts.

The third compound which is 4-(p-methoxy benzylideneamino)-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one, I(**c**) has nearly the same absorptions except the shielding effects on the p-methoxy benzenes, the four aromatic protons at 7.70-7.83 ppm and the methoxy protons at 3,81ppm. All these values were seen in (**Table 2**), and agreed with the literatures[38].

The disappearance of FT-IR bands in the region at 1700 cm^{-1} for the starting aromatic aldehyde's carbonyl group, and the appearance of the characteristic of the imine functional group at 1586 -1617 cm^{-1} in addition to the pyrazole exocyclic carbonyl bond at 1480-1485 cm^{-1} confirmed the formation of these new products[39].

The basicity of Reline, i.e. high "pH" of about 8.91 relative to acidic Malonine of "pH" of about 2.39 gave the same catalytic results on the yields percentages by increasing these yields to 95%[20].

Synthesis of pyrazol-4-yl-thiazolidin-4-one derivatives II(a-c)[36]:

After the synthesis Schiff bases materials, I (**a-c**) were effectively synthesized with stoichiometry yields, i.e. 95% with these magic deep eutectic solvents (Reline and Malonine), this was encouraged to use these catalysts in the cyclization of these Schiff bases with thioglycolic acid in the next step to synthesis the target new pyrazol-4-yl-thiazolidin-4-one derivatives II (**a-c**) with the same results of stoichiometric yields. These catalysts helped the attack of imine nitrogen atom on the carbonyl group of glycolic acid as the following suggested mechanism, (**Figure 9**).

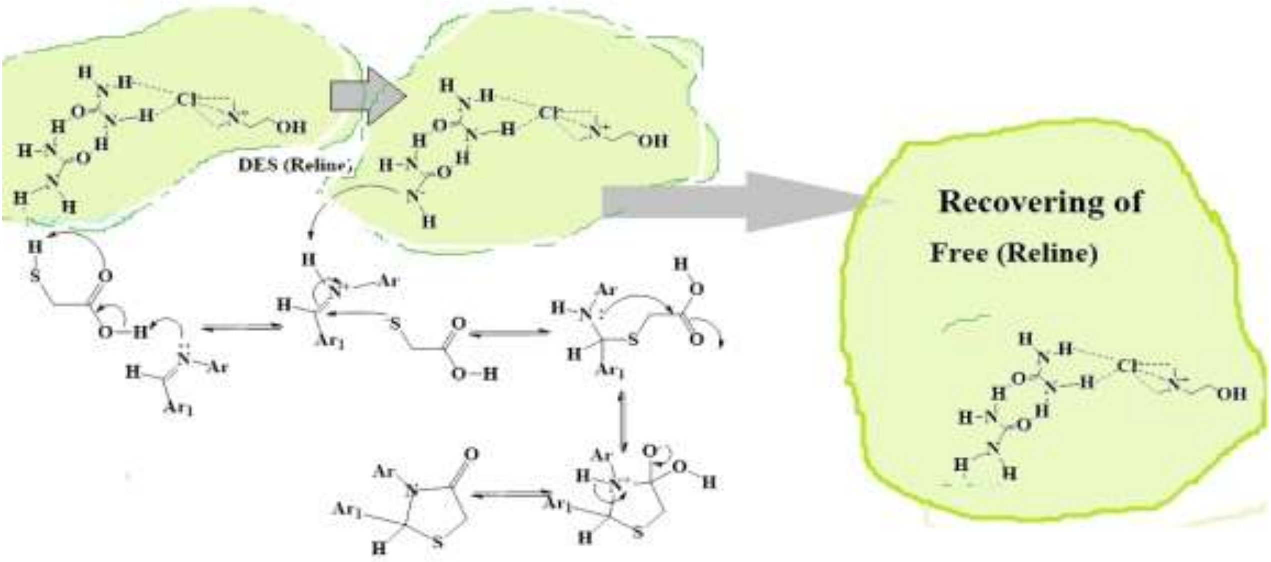

Figure 9. A suggested mechanism for the synthesis of pyrazol-4-yl-thiazolidin-4-one derivatives via Schiff bases.

3.2. Recovering of Reline deep eutectic solvent (DES)

The process of recycling Reline after each seven uses as a catalyst is economically significant, in addition to its contribution to environmental sustainability[39]. The method described in the experimental section of this paper is simple, but prolonged heating may lead to the degradation of choline chloride, which diminishes the catalytic power of Reline. This degradation occurs due to the nucleophilic bimolecular substitution (SN2) of choline chloride, resulting in the formation of trimethylamine and 2-chloroethanol, as illustrated in (**Figure 10**). To counter this, activation involves the addition of choline chloride to facilitate the in situ reformation of a new Reline deep eutectic solvent[16,25,40]. (**Figure 11**) presents the FT-IR spectrum of the recovered Reline.

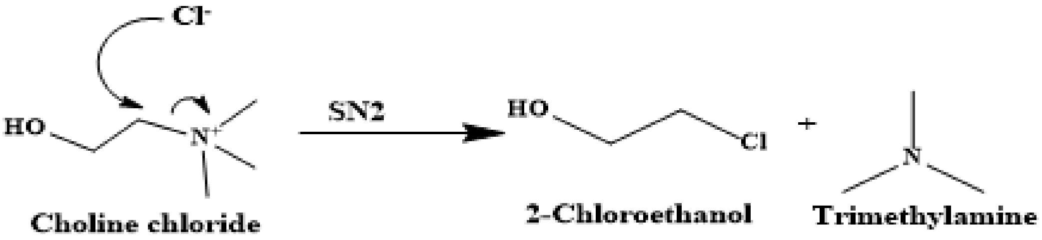

Figure 10. Degradation Mechanism of Choline Chloride.

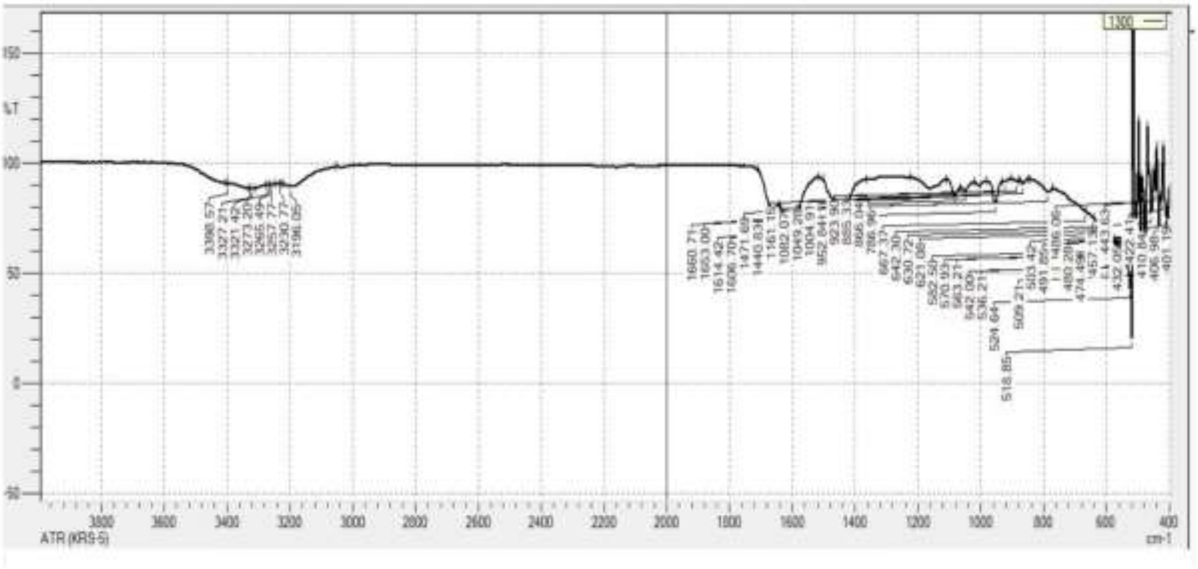

Figure 11. The FT-IR of the recovered Reline.

The yields of all these new target derivatives II (**a-c**) were also about 95%. The FT-IR spectra did not show the absorption band of the imine (C=N) group at (1595-1597 cm^{-1}) along with the appearance of a new absorption band at (1695-1680 cm^{-1}) which is imputed to (C=O) of the new thiazoledine-4-one ring, and all these absorption bands were agreed with literature[17].

^{1}H NMR (d_6-DMSO), spectra that were verified for unsubstituted 3-(3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-2-phenylthiazolidin-4-one II (**a**) was displayed distinct signals corresponding to its structural framework, as the following ^{1}H NMR spectral data :

The proton at pyrazolone ring position (5) was (δ 6.0 ppm), which resonated in this region, as a singlet because of the electronic delocalization effects, while the Pyrazolone proton(NH, δ 9.40 ppm), and it was a broad singlet, tautomeric form is present, an exchangeable NH proton may appear as a broad singlet in the downfield region due to hydrogen bonding and resonance effects. If the pyrazolone tautomeric form is present, an exchangeable NH proton may appear as a broad singlet in the downfield region due to hydrogen bonding and resonance effects, The proton at carbon position (2) of the thiazolidine ring was (5.9 ppm, singlet), and the two protons at carbon position (5) of the thiazolidinone ring appeared at (3.85-3.95 ppm) as a doublet multiplet, at with noticeable coupling to the adjacent methine proton. The two other products II (**b, c**) have the same absorption peaks except for the shielding effects on the p-methoxy benzene of compound II (**b**), the four aromatic protons at 7.70-7.83 ppm, and the methoxy protons at 3,81ppm of compound II(d). All these values were seen in (**Table 3**), and agreed with the literatures[41].

The ten aromatic protons of the two phenyl rings substituted at nitrogen number (2) of the pyrazole ring and carbon number (2) of the thiazole ring displayed a multiplet at 7.28-7.36. Also, the heterocyclic protons absorptions were :6.61(s, H, thiazole C2), 3.61 (q, 2H, thiazole C5). Finally, 9.40 (s, H, pyrazole, N1), this cyclic amine proton may be subject to H-bonding.

3.3. Structure–Antibacterial Activity Relationship (SAR)

This study focused on the synthesis of novel pyrazol-4-yl-thiazolidin-4-one derivatives II(a–c) due to their previously reported broad-spectrum antibacterial activities. The analysis of structure–activity relationships (SAR) revealed that the electron-donating substituent (–OCH_3) at the para position of compound II(c) activates the benzene ring, thereby enhancing antibacterial activity. In contrast, the electron-withdrawing substituent (–NO_2) at the meta position in compound II(b), located close to the active site, was found to reduce antibacterial efficacy.

From a mechanistic perspective, these two functional groups differ significantly in their electronic effects and hydrogen-bonding capacities. The nitro group (–NO_2) acts as a stronger hydrogen bond acceptor compared with the methoxy group (–OCH_3), owing to its highly electronegative oxygen atoms. Consequently, the nitro group increases molecular polarity and decreases electron density on the aromatic ring due to its electron-withdrawing character. Such changes can markedly influence antibacterial activity by altering membrane permeability, binding interactions with bacterial targets, and metabolic stability[42,43].

The antibacterial activities of the synthesized compounds II(a–c) were evaluated using standard in vitro methods against four pathogenic microorganisms: the Gram-positive bacteria *Bacillus cereus* and *Staphylococcus aureus*, as well as the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. In addition, antifungal screening was performed against *Candida albicans*. Penicillin was used as the reference standard for antibacterial assays[44,45].

Table 3. The antibacterial activities for compounds(a,b, and c).

	Gram -ve		***Gram +ve***	
Comp. no.	***Escherichia coli* mm/mg**	***Pseudomonas aeruginosa* mm/mg**	***Bacillus cereus* mm/mg**	***Staphylococcus aureus* mm/mg**
IIa	11	31	19	30
IIb	12	40	20	28
IIc	19	36	22	31
Penicillin	16	24	24	30

Staphylococcus aureus (31 mm/mg), surpassing the reference drug, penicillin (16 mm/mg and 30 mm/mg, respectively). Compound B showed the strongest inhibition against *Pseudomonas aeruginosa* (40 mm/mg), significantly outperforming penicillin (24 mm/mg). While penicillin itself displayed the highest activity against *Bacillus cereus* (24 mm/mg), Compound C showed comparable inhibition (22 mm/mg). Overall, these results suggest that the tested novel compounds, particularly b and c, have substantial antibacterial potential, with some outperforming penicillin against certain bacterial strains. Further investigations, including mechanistic studies and toxicity evaluations, are warranted to explore their potential as new antibacterial agents[46].

3.4. The statistical rigor of the antibacterial activities of compounds (a,b, and c)

To evaluate the statistical rigor of the antibacterial activities of compounds (**a**,**b**, and **c**), the mean, standard deviation (SD), and standard error of the mean (SEM) for each bacterial strain will be calculated. If necessary, confidence intervals will also be determined. The statistical results for *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Staphylococcus aureus* (expressed in mm/mg) are presented in (**Table 4**).

Table 4. Biological activity results.

Bacterial Strain	**Mean**	**Standard Deviation (SD)**	**Standard Error of the Mean (SEM)**
Escherichia coli	14.5	3.70	1.85
Pseudomonas aeruginosa	32.75	6.90	3.45
Bacillus cereus	21.25	2.22	1.11
Staphylococcus aureus	29.75	1.26	0.63

Note that data variability is represented by the standard deviation (SD), while the precision of the sample mean is estimated using the standard error of the mean (SEM).

3.5. The conclusions from the statistical analysis

Escherichia coli (E. coli) exhibits moderate variability in inhibition, as reflected by a relatively high standard deviation. While the standard error suggests reasonable precision, additional replicates could enhance confidence in the result.

Pseudomonas aeruginosa demonstrates the highest mean inhibition but also shows significant variability among samples, as indicated by the largest standard deviation. The relatively high standard error suggests that additional replicates would improve the reliability of this measurement.

Bacillus cereus presents a more consistent inhibition pattern, with a lower standard deviation indicating uniform results across replicates. The relatively small standard error further supports the reliability and precision of the mean.

Staphylococcus aureus exhibits the most consistent inhibition, with the lowest standard deviation suggesting minimal variability. The smallest standard error reinforces this as the most statistically precise measurement among the four bacterial strains.

4. Conclusion

This work represents a green synthesis approach, utilizing deep eutectic solvents (DESs), Reline and Malonine, as green solvents. These DESs were easily prepared by combining choline chloride with either urea or malonic acid in a molar ratio of 1:2 and 1:1, respectively. Serving as dual-function catalysts with low melting points and excellent solvent properties for organic reactions, they also offer advantages such as affordability, non-toxicity, biodegradability, and renewability.

The use of these DESs significantly enhanced the percentage yields of Schiff bases, 1,5-dimethyl-4-(substituted styryl)-2-phenyl-1,2-dihydro-3H-pyrazol-3-one (**I-a–c**), achieving a nearly stoichiometric yield (95%) in methods B and C, compared to the 76–80% yield obtained through the conventional synthesis method (A). Additionally, reaction times were substantially reduced to 30 minutes, as opposed to 120 minutes in method A. Similarly, in methods E and F, solvent-free conditions, simplified procedures, and a stoichiometric yield (95%) were achieved, contrasting sharply with the 50–51% yield observed in the conventional method (D). Furthermore, reaction times in methods E and F were reduced to a quarter of those in method D during the synthesis of the novel compounds II (**a–c**). Finally, *Pseudomonas aeruginosa* exhibited the highest inhibition; however, the large variation suggests that further testing is needed to ensure confidence in the results. *Staphylococcus aureus* demonstrated highly consistent inhibition values, making its measurements the most reliable. *Escherichia coli* showed the lowest inhibition, with moderate variability, while *Bacillus cereus* displayed stable results, indicating a reproducible effect.

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Data Availability Statement

Applicable.

Conflict of interest

The authors declare no conflict of interest.

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