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Green antibacterial approach: Ultrasound-enhanced date seed (*Phoenix dactylifera* L.) extracts against *Escherichia coli*

Alanood A. Alfaleh[1,2], Marya J. Hamdan[1], Abdullatif A. Neamatallah[2], Nagwa T. Elsharawy[*3]

[1]*Department of Food and Nutrition, Faculty of Human Sciences and Design, King Abdulaziz University, Jeddah, Saudi Arabia*

[2]*Department of Environmental Sciences, Faculty of Environmental Sciences, King Abdulaziz University, Jeddah, Saudi Arabia*

[3]*Department of Movement Science & Health, Collage of Sport Science, University of Jeddah, Jeddah, Saudi Arabia*

[4]*Department of Food Hygiene, Faculty of Veterinary Medicine, New Valley University, Egypt*

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Abstract

Natural Deep Eutectic Solvents (NADES) are novel greener and sustainable solvents that enhance phenolic compound extraction from different plant sources. The study aims to extract date seeds using three natural solvents and ultrasound and test the antibacterial effect of date seeds extracts against *Escherichia coli* bacteria by using "the well diffusion" method to measure the zone of inhibition (ZOI). The results show that the date seeds extracted with NADES such as; lactic and citric acids significantly exhibited the highest ZOI values (40.5 ± 0.71 and 36.0 ± 2.83 mm, respectively) ($p < 0.05$) compared to the standard antibiotic Ciprofloxacin (29.5 ± 0.71 mm) and other date seeds extracts. Also, it found that each date seeds extract significantly exhibited ($p < 0.05$) higher ZOI values than its extracted solvent. The study recommends that the extraction process enhance the antibacterial properties of the extracts. These findings noted that NADES containing carboxylic acids have the potential to extract polyphenols from date seeds with notable antibacterial activities. NADES can be utilized in various industries such as food and pharmaceuticals as environmentally friendly solvents.

* **Corresponding Author:** Nagwa T. Elsharawy ✉ dr.nagwa2004@yahoo.com

Introduction

Bioactive compounds are extracted from plants using various traditionally aqueous-organic solvents, including ethanol, methanol, chloroform, and acetone. However, this conventional technique has several limitations, such as the toxicity of the solvents, thermal instability of the compounds, and low recovery rates (Dheyab *et al.*, 2021; Jurić *et al.*, 2021). To address these issues and reduce the negative environmental impact, the development of extraction solvents with fewer drawbacks has become increasingly important.

The use of green solvents, which possess attributes like sustainability, biodegradability, low volatility, low toxicity, low cost, and simple preparation methods has garnered considerable interest as a substitute for traditional organic solvents (Xin *et al.*, 2017; Zannou and Koca, 2022). Among green solvents, natural deep eutectic solvents (NADES) have garnered significant interest from researchers (Dai *et al.*, 2013; Bakirtzi *et al.*, 2016). Utilizing NADES as a solvent for extraction could improve the solubility and bioactivity of bioactive substances in comparison to traditional solvents (Li, 2022). They are liquid mixtures formed through the hydrogen-bonding interaction of two or more natural components of essential metabolites such as alcohols, sugars, amino acids, and organic acids (Fuad *et al.*, 2021).

Although NADES have mainly been studied for their applications in chemistry, pharmaceuticals, and food processing, researchers have also examined their antimicrobial properties to a certain extent (Pavić *et al.*, 2019; Jurić *et al.*, 2021). The antimicrobial activity of NADES extractants, which includes the capability to prevent bacteria and fungi from growing, is significant. This characteristic is particularly valuable in the development of new, environmentally friendly, and efficient solvents, especially in the nutraceutical and pharmaceutical industries. NADES extracts can preserve and enhance the biological activities of the extracted compounds (Grozdanova *et al.*, 2020; Fuad *et al.*, 2021).

Multiple investigations have examined the antimicrobial properties of date (*Phoenix dactylifera* L.) seeds extract obtained from various crops and types (Metoui *et al.*, 2019; Barakat *et al.*, 2020; Maqsood *et al.*, 2020). These studies have primarily focused on assessing the effectiveness of date seeds extracts, obtained using conventional solvents like water and organic solvent (methanol, ethanol, acetone), concerning the antibacterial activity of different types of bacteria including *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The findings suggest that most date seeds extract exhibit antibacterial properties against a range of bacterial strains.

According to Platat *et al.* (2014) and Alkhoori *et al.* (2022), date palm (*Phoenix dactylifera* L.) seeds have a high concentration of polyphenols, and the presence of these bioactive compounds contributes to their numerous health benefits, including antibacterial activity. Also, these compounds are widely used to replace synthetic additives and enhance the quality of food products (Dheyab *et al.*, 2021). Papuc *et al.* (2017) noted that the polyphenols in date seed extracts can potentially interact with microbial cell walls and intracellular structures, leading to disruptions in their growth and functionality.

Despite being regarded as environmentally safe and sustainable alternatives to traditional solvents, the use of NADES in extracting polyphenols from date seeds has been subject to limited investigation. Furthermore, NADES can be formulated in an almost infinite number of combinations, but their influence on the properties of the resulting extract, particularly in terms of antibacterial activity, is largely unidentified. Therefore, this study aimed to examine the antibacterial activity effectiveness against *Escherichia coli* bacteria of date seeds extracted with three prepared NADES using an ultrasonic extraction technique.

Materials and methods

Samples and materials

Date seeds of the Khalas variety produced in the year 2021 were obtained as a by-product from three randomly chosen date factories throughout the Kingdom of Saudi Arabia. L-proline (Pro) (≥99.0%) was purchased from (Solarbio Science and

Technology, Beijing, China). L (+)-lactic acid (La) (95.0%) was provided by (PanReac AppliChem, Barcelona, Spain). Citric acid (Citr) (99.0%), glycerol (Gly) (≥99.0%), HPLC grade ethanol (EtOH), and Ciprofloxacin were acquired from (Sigma-Aldrich, St. Louis, MO, USA). A bacterial strain of *Escherichia coli* (ATCC 25922) was provided by a laboratory of microbiology (King Abdulaziz University Hospital, Jeddah, KSA).

Methods

Natural deep eutectic solvents (NADES) preparation

The NADES preparation method followed the procedure described by Karadendrou *et al.* (2022). In Table 1, L-proline was used as a hydrogen bond acceptor (HBA), while carboxylic acids (lactic and citric acids) and alcohol (glycerol) served as hydrogen bond donors (HBD). The molar ratios of the components used were Pro-La (1:2), Pro-Citr (1:1), and Pro-Gly (1:2). Subsequently, all three NADES were diluted with 20 or 30% (v/v) distilled water.

Chemical and physical properties of NADES

All 3 NADES samples were evaluated using pH and viscosity properties. The pH measurements were conducted at 24 °C using a pH meter (Jenway, UK). The viscosity of the low-viscosity NADES (Pro-La) was determined using strain S61, while the high-viscosity NADES (Pro-Citr and Pro-Gly) were tested using strain S62. The viscosity measurements were carried out at a speed of 30 rpm and a temperature of 24 °C using a viscometer (Brookfield, USA).

Date seeds sample preparation

The date seeds were randomly homogenized, cleansed to remove any remaining date flesh, and then dried in an oven (Memmert, Western Germany) at a temperature of 50 °C for 12 h. A heavy-duty grinder (Alsaif-Elec, KSA) was used to grind the seeds initially. Subsequently, the seeds were finely ground into a powder with a particle size of 0.5 mm (Platat *et al.*, 2014; Ben-Youssef *et al.*, 2017).

Date seeds powder extraction

The extraction of date seeds followed the procedure described by Ben-Youssef *et al.* (2017). The powdered date seeds were mixed with the prepared NADES (Pro-La, Pro-Citr, or Pro-Gly) or conventional solvents (water or 70% ethanol) at a ratio of 1:10 (g/ml). The mixtures were then transferred to a sonication water bath (HumanLab, Korea) and subjected to a temperature of 40 °C for 30 min. After sonication, five extracted samples were centrifuged for 10 min at 3900 rpm using a centrifuge (Sigma, Germany) and secured at -20 °C until further experiments.

Antibacterial activity determination

The antibacterial activity of five date seeds (DS) extracts (DS-water, DS-EtOH 70%, DS-Pro-La, DS-Pro-Citr, DS-Pro-Gly) and their solvents (water, EtOH 70%, Pro-La, Pro-Citr, Pro-Gly) was tested against *Escherichia coli* using Agar well diffusion method according to Jahangirian *et al.* (2013).

The bacterial cultures were preserved at a temperature of -80 °C in Mueller-Hinton broth (MHB) until the subculturing of the Bacteria. *Escherichia coli* were prepared according to Ivanović *et al.* (2022). The cultures were placed on Petri dishes containing Mueller-Hinton agar (MHA) and kept in an incubator at a temperature of 37 °C for duration of 24 h. Then, three to five separate colonies were moved to a tube (5 ml) comprising MHB and incubated within a time frame of 2 to 6 h. The bacteria suspensions were standardized to 1.5 × 108 CFU/ml, and the optical density (OD) of the colony suspension was measured using a spectrophotometer at a wavelength of 620 nm using the 0.5 McFarland standard for contrast.

To evaluate the antibacterial characteristics, holes (6 millimeters) were created in the microbial plates and filled with 100-µl of date seeds extracts and their solvents, and a standard antibiotic (Ciprofloxacin 5 µg/ml) was used as a positive control (ve+). The dishes were afterward placed in an incubator for 24 h at a temperature of 37 °C. Following this, the zone of inhibition (ZOI) was measured as the absence of bacterial growth surrounding the holes and was measured by millimeter (mm) using a caliper, and

tests were performed in duplicate. The halos were categorized as follows: susceptible with a diameter of 21 mm or greater, intermediate with a diameter between 16 and 20 mm, and resistant with a diameter of 15 mm or less according to CLSI (2016).

Statistical analysis

The experimental data was analyzed using IBM SPSS statistics software version 25.0. The data were reported as the average of duplicate measurements with the ± standard deviation (SD) indicated. The data was subjected to a one-way analysis of variance (ANOVA) to conduct statistical analysis. The significance of the variations between means was determined using Duncan's multiple-range test (DMRT). Statistical significance was assessed based on differences with a p-value less than 0.05.

Results

The pH and viscosity values obtained are shown in Table 1. The pH measurements showed clear differences between the three investigated NADES. Pro-La had a significantly lower pH of 2.69 ± 0.01, suggesting a highly acidic composition. Similarly, Pro-Citr had a pH of 2.11 ± 0.03, reinforcing its strong acidity. On the other hand, Pro-Gly had a pH of 6.64 ± 0.03, indicating a nearly neutral nature. This indicates that Pro-La and Pro-Citr have notably acidic properties compared to Pro-Gly. The viscosity levels also exhibited distinct variations among the NADES. Pro-La had a lower viscosity of 65.67 ± 0.12 cP, which was significantly different from the other solvents. In contrast, Pro-Gly demonstrated a much higher viscosity of 246.0 ± 1.0 cP, while Pro-Citr showed the highest viscosity among the NADES, with a value of 308.0 ± 1.0 cP, indicating a significantly thicker consistency.

Table 2 shows the comparative analysis of the antibacterial activity that was conducted between the standard antibiotic Ciprofloxacin (5 µg/ml), NADES, conventional solvents, and date seeds extracts against *Escherichia coli* bacteria. The results were defined as the zone of inhibition (ZOI), represented as the mean value ± standard deviation (SD) in millimeters. Statistical significance was determined at a p-value of less than 0.05.

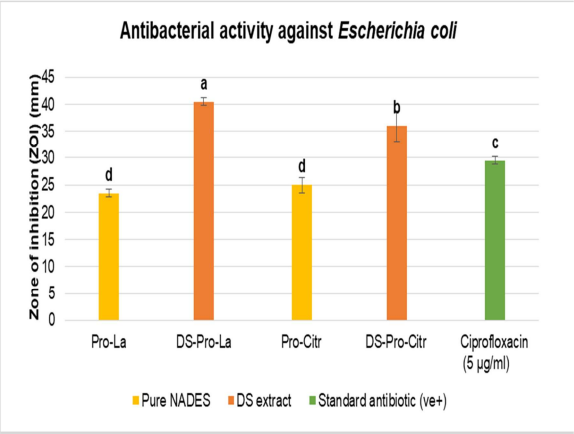

Fig. 1. Antibacterial activity of date seeds extracted with carboxylic acids-based NADES against *Escherichia coli*. NADES: natural deep eutectic solvents; Pro: L-proline; La: lactic acid; Citr: citric acid; DS: date seeds; ve+: positive control; mm: millimeter. The outcomes were described as a diameter of ZOI (mm). Each value represents the mean of 2 ± SD. Dissimilar letters express significant differences with *p*-value < 0.05.

Regarding *Escherichia coli* bacteria, to begin with solvents, when comparing the standard antibiotic Ciprofloxacin as the positive control (ve+) with the group of solvents, it found that Pro-Citr and Pro-La exhibited inhibition zones of 25.0 ± 1.41 and 23.5 ± 0.71 mm, respectively, which were significantly lower ($p < 0.05$) compared to the inhibition zone of Ciprofloxacin (29.5 ± 0.71 mm). These results indicate that Pro-Citr and Pro-La have antibacterial activity against *Escherichia coli* bacteria despite being less effective than the Ciprofloxacin as shown in Fig. 1 and Fig. 2 (E, G, K).

Fig. 2 (A, C, E, G, I) showed a comparison of conventional solvents (water and EtOH 70%) with NADES, it noticed that the conventional solvents in addition to Pro-Gly NADES did not exhibit any antibacterial activity for *Escherichia coli*. While Pro-Citr and Pro-La respectively, showed efficacy as an antibacterial against *Escherichia coli* bacteria with ZOI > 21 mm.

Table 1. The abbreviations, compositions, molar ratio, appearance, and properties of NADES

Abbreviations	Composition		Molar ratio	Formed color	pH	Viscosity cP
	HBA	HBD				
Pro-La	L-proline	lactic acid	1:2	Yellow transparent liquid	2.69 ± 0.01	65.67 ± 0.12
Pro-Citr	L-proline	citric acid	1:1	Light brown transparent liquid	2.11 ± 0.03	308.0 ± 1.0
Pro-Gly	L-proline	glycerol	1:2	Orange-yellow transparent liquid	6.64 ± 0.03	246.0 ± 1.0

NADES: natural deep eutectic solvents; HBA: hydrogen bond acceptor; HBD: hydrogen bond donor; L-proline (Pro); lactic acid (La); citric acid (Citr); glycerol (Gly); cP: Centipoise.

Table 2. Antibacterial activity of NADES and date seeds extracts against *Escherichia coli*

Samples		Antibacterial activity (ZOI) (mm) against *Escherichia coli*
Conventional solvents	water	n.d
	EtOH 70%	n.d
NADES	Pro-La	23.50 ± 0.71 ^{d}
	Pro-Citr	25.00 ± 1.41 ^{d}
	Pro-Gly	n.d
Conventional extraction	DS-water	n.d
	DS-EtOH 70%	n.d
NADES extraction	DS-Pro-La	40.50 ± 0.71 ^{a}
	DS-Pro-Citr	36.00 ± 2.83 ^{b}
	DS-Pro-Gly	17.00 ± 1.41 ^{e}
Antibiotic (ve+)	Ciprofloxacin	29.50 ± 0.71 ^{c}

NADES: natural deep eutectic solvents; Pro: L-proline; La: lactic acid; Citr: citric acid; Gly: glycerol; EtOH: ethanol; DS: date seeds; ve+: positive control; ZOI: zone of inhibition; mm: millimeter; n.d: not detected. The outcomes were described as a diameter of ZOI (mm). Each value represents the mean of 2 ± SD. Dissimilar letters express significant differences with p-value < 0.05.

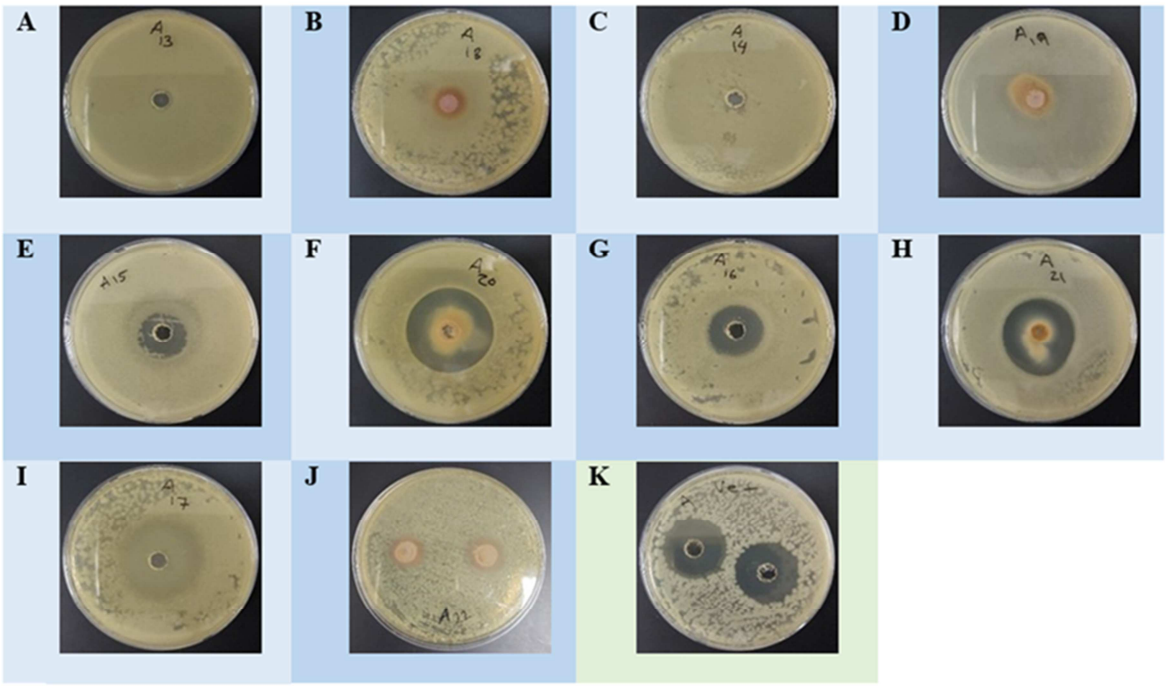

Fig. 2. Zone of inhibition for the solvents, date seeds extracts, and standard antibiotic, against *Escherichia coli*
A: water, B: DS-water, C: EtOH 70%, D: DS-EtOH 70%, E: Pro-La, F: DS-Pro-La, G: Pro-Citr, H: DS-Pro-Citr, I: Pro-Gly, J: DS-Pro-Gly, K: Ciprofloxacin

The effectiveness of different date seeds extracts as antibacterial agents against *Escherichia coli* bacteria was compared to that of Ciprofloxacin. The results in Fig. 1 showed that DS-Pro-La exhibited the highest efficacy with a ZOI measuring 40.5 ± 0.71 mm, followed by DS-Pro-Citr with a ZOI of 36.0 ± 2.83 mm. There was a significant difference observed between these two extracts ($p < 0.05$). Importantly, the effectiveness of Ciprofloxacin (29.5 ± 0.71 mm) was significantly lower than both DS-Pro-La and DS-Pro-Citr ($p < 0.05$) as shown in Fig. 1 and Fig. 2 (F, H, K). Additionally, DS-Pro-Gly demonstrated a ZOI of 17.0 ± 1.41 mm, but it was significantly less effective than the Ciprofloxacin ($p < 0.05$) as presented in Fig. 2 (J, K).

Fig. 2 (B, D, F, H, J) showed that conventional solvent extracts (DS-water and DS-EtOH 70%) did not have any bacterial inhibition ability against *Escherichia coli* while the efficacy of bacterial inhibition exceeds in DS-Pro-La, DS-Pro-Citri, and DS-Pro-Gly, respectively. Moreover, when comparing the ZOI value for all date seeds extracts with their respective solvents, the results showed that each date seeds extract had a higher inhibition zone value than its solvent, with significant differences ($p < 0.05$) as presented in Fig. 2 (E-J).

Discussion

The proficiency of NADES extractants to impede the maturation of microorganisms, namely bacteria and fungi, cannot be undervalued. This characteristic holds great value in the development of a new, environmentally friendly, and effective solvent, particularly within the nutraceutical and pharmaceutical sectors by their ability to preserve and enhance the biological properties of extracted bioactive compounds (Fuad *et al.*, 2021).

In the present study, the effects of pure NADES and date seeds extracts obtained using NADES on the antibacterial activity against *Escherichia coli* were investigated. The impact of these substances was assessed using the well diffusion method. When comparing the group of solvents, which included both conventional solvents and pure NADES, with the standard antibiotic Ciprofloxacin, the results indicated that NADES containing carboxylic acids (Pro-La and Pro-Citr) exhibited higher ZOI values that were similar to those of Ciprofloxacin against *Escherichia coli*. The presence of organic acids in NADES may contribute to their antimicrobial properties, as organic acids are known to have antimicrobial effects.

These findings are in agreement with Radošević *et al.* (2018) who examined the antibacterial activity of ten NADES against several bacterial strains (*Staphylococcus aureus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Escherichia coli*). The results indicated that the antibacterial activity of the tested NADES varied depending on their composition. Only NADES containing organic acids showed toxicity towards the bacterial strains. This suggests that the individual components of NADES can influence their overall antibacterial properties.

In the study conducted by Jurić *et al.* (2021), the researchers investigated the antibacterial properties of six different types of NADES against various bacterial strains, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella enterica*. The microdilution method was used to assess the inhibitory effects of NADES on bacterial growth, and the results were compared to those of 70% ethanol. The study revealed that all examined NADES exhibited a higher degree of growth of bacteria inhibition at lower concentrations when compared to 70% ethanol. Moreover, the results of the study displayed that NADES containing a carboxyl group in their composition exhibited significant antibacterial efficacy against all four bacterial strains examined in the study.

The possible explanation for the antibacterial properties revealed by carboxylic acids-based NADES can be imputed to their ability to induce an acidic environment within the medium. This corresponds with the previous study that emphasized the ability of

organic acids to lower the pH and disrupt the proton motive force across the bacterial cell membrane (Radošević *et al.*, 2018). This finding suggests that the existence of organic acids in NADES can disrupt the integrity of the bacterial cell membrane or interfere with essential cellular processes, leading to bacterial growth inhibition or cell death. According to Madigan *et al.* (2008), the acidic environments created by NADES can impede the action of bacterial enzymes that have a vital role in various biological functions. Also, bacterial enzymes have optimal pH ranges for their activity, and deviations from these ranges can lead to enzyme breakdown or reduced activity. The observed findings showed the highest pH value of Pro-Citr (2.11 ± 0.03), followed by Pro-La (2.69 ± 0.01), which interprets the exceeding of ZOI value in Pro-Citr NADES.

Furthermore, Ivanović *et al.* (2022) stated that the high density and viscosity of NADES can impede their diffusion from the disk into the surrounding environment. This may potentially influence the observed interaction between NADES and cells, as the limited diffusion could affect the extent of contact between NADES and the target cells. According to that, the lower viscosity of Pro-La (65.67 ± 0.12 cP) compared to other NADES in the current study may affect its spreading within the medium, which demonstrated its high impact on bacterial growth inhibition. However, Pro-Citr showed the highest viscosity among the studied NADES of 308.0 ± 1.0 cP, despite exhibiting the highest efficacy of the inhibition zone against *Escherichia coli*, which can be attributed to its lower pH level as described earlier.

In the present study, no antibacterial activity was observed for the Pro-Gly NADES. This result is compliant with the outcomes reported by Zhao *et al.* (2015), in which NADES containing alcohols as HBD did not exhibit inhibitory effects on the growth of *Staphylococcus aureus* and *Escherichia coli*. It was expected that the glycerol substance studied would exhibit less toxicity towards bacteria. This is because many microorganisms can utilize certain composites such as glycerol as sources of nutrition (Silva *et al.*, 2010; Juneidi *et al.*, 2016).

Alkhoori *et al.* (2022) observed that date seeds contain a high concentration of polyphenols, which contribute to their numerous health benefits, including antibacterial activity. The current investigation found that the date seeds extracted with NADES displayed a significant effect on the antibacterial activity exceeding the antibiotic Ciprofloxacin and their pure NADES, particularly the one extracted with the carboxylic acid-based NADES. The findings might be a result of the interactions between polyphenols and bacteria suggested by Papuc *et al.* (2017), through which they have noted that the polyphenols present in date seed extracts can potentially interact with microbial cell walls and internal structures, causing disruptions in their growth and functionality. Polyphenols can exhibit antibacterial properties, inhibiting the growth of certain bacteria. They can disrupt bacterial cell membranes, interfere with essential enzymes, and inhibit bacterial adhesion and biofilm formation (Makarewicz *et al.*, 2021).

A study conducted by Pavić *et al.* (2019) evaluated the antibacterial efficacy of the rue leaf extracts using NADES against various bacterial strains, particularly focusing on gram-negative *Pseudomonas aeruginosa*. The findings demonstrated that the rue extract showed the highest antibacterial efficacy against all the strains that were tested. The antibacterial efficacy of the rue extracts can be attributed to the existence of bioactive components, including furanocoumarins, alkaloids, and phenolic compounds. These compounds have been previously reported to possess antimicrobial properties.

Conclusion

The study demonstrated that NADES outperformed the conventional solvents in terms of enhancing antibacterial activity in date seeds. NADES composed of L-proline with carboxylic acids (Pro-La and Pro-Citr) exhibited the highest antibacterial activity along with their extracts compared to other solvents. By replacing traditional hazardous solvents with NADES, sustainable extraction methods can be developed, aligning with the principles of green consumerism. This approach utilizes date seeds as a valuable source

of polyphenols with various health benefits. Extracting these compounds using NADES will provide the potential for developing nutraceutical and functional food products. However, the study had some limitations, including its focus on the limited number of NADES formulations and microorganisms.

Recommendation(s)

Further analysis of polyphenolic compounds is recommended to identify and quantify polyphenols present in NADES extracts, which will enhance understanding of their contribution to the observed antibacterial activity. Also, future research could investigate different types of NADES formulations to optimize the extraction process and evaluate their feasibility in industrial settings, aiming to improve human health and well-being.

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