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Research

Unravelling The Bioactivities of *Acmella paniculata* Extract-Mediated Green Deep Eutectic Solvent of Citric Acid Monohydrate and Glycerol

Rajina Shahmir Sivaraj[1], Roziana Mohamed Hanaphi[1]*, Rizana Yusof[2]

1. Department of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA Perlis Branch, Arau Campus, 02600 Arau, Perlis, Malaysia
2. Department of Chemistry, Faculty of Applied Sciences, Universiti Teknologi MARA Perlis Branch, Arau Campus, 02600 Arau, Perlis, Malaysia
*Corresponding author: roziana@uitm.edu.myy

ABSTRACT

Plants are important sources of underlying medicinal value properties. The extraction of bioactive compounds from botanical sources using green solvents has gained interest due to its environmental sustainability. This study highlighted the bioactivities potential of *Acmella paniculata* extract mediated by green deep eutectic solvent (DES) composed of the citric acid monohydrate and glycerol. *Acmella paniculata*, a local flowering shrub was selected due to its rich medicinal value compounds. The synergistic effect between plant's bioactive compounds and DES is capable of enhancing bioactivity, making DES a promising plant solvent extractor candidate. The plant extracts were prepared in leaf and flower parts using the centrifugation method. The phytochemical screening for both extracts showed the presence of terpenoids and steroid constituents which have valuable bioactivity functions. The antibacterial activity assessed by disc diffusion assay exhibited higher susceptible bacterial response of *E. coli, Salmonella enterica* ser. Typhimurium and *S. aureus* against the flower extract compared to the leaf extract. The DPPH assay was conducted to assess free radical scavenging activity. The flower extract demonstrated radical scavenging activity (RSA) of 75%-77% while the leaf extract demonstrated 65%-69%. The flower extract results showed higher RSA emphasizing its potential as a natural antioxidant. The anti-inflammatory activity was determined by egg albumin denaturation assay, which showed a greater inhibition rate in flower extract than the leaf extract which was up to 95% and 89% respectively. Thus, both extracts possess an *in vitro* anti-inflammatory effect. Conclusively, flower extract exhibited better bioactivities value than leaf extract in a green DES. Hence, offering a new insight into its application as an effective alternative in natural product-based therapeutics.

Key words: *Acmella paniculata*, bioactivities, deep eutectic solvent, green solvent, plant extract

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INTRODUCTION

Acmella paniculata also known as *Spilanthes acmella, Sthenodesme temtanara,* and *Spilanthes paniculata* is usually referred to as "Subang nenek" or toothache plant (Panyadee & Inta, 2022). *Acmella paniculata* is one of the species that is abundantly found all over the world such as in Brazil, Bangladesh, Cambodia, China, Jawa, Peru, North Australia, Borneo, Malaysia, Indonesia, India, Thailand, Sri Lanka, and more (Rahim *et al*., 2021; Plants of the World Online, n.d.). Specifically, this small-sized shrub can be found in damp areas such as swamps, ponds, or roadside (Rahim *et al*., 2021; Panyadee & Inta, 2022). In Malaysia, this plant has been used locally as an alternative remedy to treat toothache and mouth ulcers by chewing the flower head (Rani *et al*., 2019). This plant consists of main contributors like spilanthol and acmellonate that decrease toothaches, stimulate saliva secretion, and create an anesthetic effect (Savant & Kareppa, 2022). Bioactive compounds are commonly extracted through conventional methods like Soxhlet extraction, soaking, and maceration, with common organic solvents such as methanol, ethanol, and hexane.

This paper explores an alternative plant extraction method using deep eutectic solvent (DES). Our previous study has implemented this DES-based extraction method, promoting its efficacy in extracting bioactive compounds from plants (Sivaraj *et al*., 2023).

DES was originated by Abbott *et al*. (2003) and is a liquid solvent consisting of a hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD). The main interactions formed between HBA and HBD are usually hydrogen bonds, occasionally electrostatic forces, and *van der Waals* interactions. The components of DES are altered by modifying the molar ratio and types of HBAs and HBDs resulting in a larger range of applications in pharmaceutical and cosmetic-related industries. Most importantly its ability as a solvent extractor has recently gained attention (Tebbi *et al*., 2023). DES has been more favorable for extraction processes, especially for bioactive compounds from plants as it is environmentally friendly, easily synthesized, and thermally stable (Gajardo-Parra *et al*., 2019). In addition, DES has sparked promising antibacterial activities against pathogenic bacteria, thus bringing potential to biomedical and health industries (Filip *et al*., 2022; Akbar *et al*., 2023).

In this study, citric acid monohydrate (CA) was chosen as the HBA as it is an organic acid with a higher number of carboxylic groups (one hydroxyl and three carboxylic acid groups) than other organic acids (lactic acid & malic acid). Therefore, it increases the overall stability of DES as it produces more hydrogen bonds, which is one of the main interactions required during synthesis (Dai *et al*., 2013; Hikmawanti *et al*., 2021; Swebocki *et al*., 2023). On the other hand, the chosen HBD was glycerol (Gly), which is a trihydric alcohol featuring three hydroxyl groups. This study highlighted the bioactivities potential of *Acmella paniculata* extract mediated by green deep eutectic solvent composed of the citric acid monohydrate and glycerol through antibacterial, antioxidant, and anti-inflammatory tests.

MATERIALS AND METHODS

Materials

Citric acid 1-hydrate (99.5-100%), glycerol, L(+)-ascorbic acid, (Hmbg, Germany) were utilized for eutectic synthesis and bioactivity tests. Phosphate buffer solution (R&M Chemicals, Malaysia), gallic acid, n-hexane, potassium iodine, hydrochloric acid (37%) and concentrated sulphuric acid (98%) (Merck, UK) were for phytochemical screening. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) powder (Sigma Aldrich, USA) was used for the antioxidant test. Nutrient broth, antibiotic discs, blank disc (Oxoid, UK), and Mueller Hinton agar (MHA) (HiMedia, India) were for antimicrobial susceptibility tests. Diclofenac sodium (Hovid, Malaysia) was used in the anti-inflammatory test.

Preparation of deep eutectic solvent (DES)

The DES was prepared by constantly heating and stirring the hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) in a molar ratio of 1:2 at 80°C until the homogenous solvent was obtained (Wu *et al*., 2021). Citric acid monohydrate (CA) was used as the HBA while glycerol (Gly) was used as HBD, thus resulting in an alternative green solvent known as DES through the combination of CA/Gly. The molar ratio shows that the higher hydroxyl groups from HBD may result in more hydrogen bonds and higher thermal stability (Basaiahgari *et al*., 2018). According to Yusoff *et al*. (2023), the melting point was determined with differential scanning calorimetry (DSC) analysis on a similar eutectic solvent. It was observed that the melting point was 150.3±0.11. The eutectic solvent was then prepared in three different concentrations (70%, 60%, 50% (v/v)) by diluting in deionized water.

The physicochemical performance of DES had been done previously before this study. Properties that include viscosity, density, solubility, polarity, and others are parameters that can affect the eutectic composition and determine its suitability for extracting bioactive constituents (Shafie *et al*., 2019). Therefore, the addition of water plays a significant role because it alters the overall characteristics by affecting the polarity and viscosity in terms of interaction between the HBA and HBD components which influences the efficacy of the solvent (Ling & Hadinoto 2022).

Plant identification of *A. paniculata*

The living specimen of *A. paniculata* was collected and cultivated in the Flora Garden of the School of Biological Sciences, Universiti Sains Malaysia (USM). It was then authenticated by a taxonomist at the Herbarium of the School of Biological Sciences, USM. The voucher specimen (USMP 11924) was kept at USM for proper preservation purposes.

Preparation of *A. paniculata*

Acmella paniculata flowers and leaves were selected and separated for analysis. The plants were

washed from soil residues and air-dried. The parts (flower & leaf) were then dried in the oven for 24 hr at a temperature below 40°C. The dried plant parts were separately ground with a blender into fine powder and stored in bottles respectively at room temperature until further use.

Extraction of *A. paniculata* using DES

The method used was according to Wu *et al*. (2021) with slight modifications. The concentration of plant extracts was initially prepared at 20 mg/mL in different DES concentrations, respectively. The plant extracts were prepared by dissolving the dried plant part with three different concentrations of DES (70%, 60%, 50% (v/v)) respectively. Each solution contained 0.2 g dried powdered *A. paniculata* in 10 mL of DES. Three solutions of DES-flower (70% DES-flower, 60% DES-flower & 50% DES-flower) and three solutions of DES-leaf (70% DES-leaf, 60% DES-leaf & 50% DES-leaf) were obtained. All six solutions were vortexed and then soaked for 2 hr. Next, the solutions were centrifuged at 5,000 rpm for 5 min. The sample solutions were filtered using gravity filtration where the extract residues were discarded. The solutions were centrifuged again and the final supernatants were collected for further testing.

Phytochemical screening of *A. paniculata* DES-based extract

The qualitative analysis was performed based on different types of bioactive compounds that may be present or absent in both DES extracts of flower and leaf.

Detection of tannins

The analysis of tannins was based on Braymer's test with some modifications. Briefly, 2 mL of the extract was stirred with 2 mL of distilled water. Three drops of 10% ferric chloride were added. The formation of a blue-black, green, or blue-green precipitate showed the presence of tannins (Oloya *et al*., 2022).

Detection of steroids

This detection of steroids was conducted using Salkowski's test with some modifications. Concisely, 0.2 mL of the extract was dissolved in 2 mL of chloroform. Concentrated sulphuric acid was added carefully to form a lower layer. A reddish-brown color at the interphase indicated the presence of steroids (Oloya *et al*., 2022).

Detection of terpenoids

The detection of terpenoids test was done according to Oloya *et al*. (2022) with some modifications. A total of 5 mL of the extract was added to 2 mL of chloroform and 3 mL of concentrated sulphuric acid. The presence of terpenoids was determined if a reddish-brown color was formed.

Detection of alkaloids

The detection of terpenoids test was done according to Shaikh and Patil (2020) with some modifications. Briefly, 2 mL of the extract was mixed carefully with 3 drops of freshly prepared Wagner's reagent (1.27 mL iodine + 2 g potassium iodide + distilled water to make a final volume of 100 mL). The formation of a brown precipitate indicated the presence of alkaloids.

The determination of *in vitro* test for *A. paniculata* DES based-extract

Antibacterial testing for *A. paniculata* DES based-extract

Kirby Bauer disc diffusion method

This antibacterial test was performed using the Kirby-Bauer disc diffusion method according to the standard protocol based on the Clinical Laboratory Standards Institute (CLSI). The antibacterial activity of the plant-DES extracts was tested against four pathogenic bacteria which were Gram-positive (*Bacillus subtilis* (ATCC 11774) and *Staphylococcus aureus* (ATCC 33592) and Gram-negative (*Escherichia coli* (ATCC 25922) and *Salmonella enterica* serovar Typhimurium (WDCM 00031)). One positive control disc (gentamicin), one negative control disc (deionized water), and three test discs containing three samples of flower-DES extract (70% DES-flower, 60% DES-flower & 50% DES-flower). The procedure was repeated for the leaf-DES extracts. A set of tests for different concentrations of DES (70%, 60% & 50% (v/v)) was prepared to determine the sole potential of DES in antibacterial activity. A fresh, sterile cotton-tipped swab was dipped into the bacterial suspension. The excess liquid was removed by

pressing it against the tube wall. It was then swabbed side to side covering the entire surface of MHA. The plate was rotated approximately 60° and repeated three times. The discs were placed within 15 min on the bacterial-inoculated MHA plate one at a time and labeled accordingly. Each disc was pressed down firmly to ensure sturdy contact with the agar. The inhibition zone diameter for each disc was measured after 24 to 48 hr of incubation at 35°C ± 2°C (Cavalieri *et al*., 2005; Kurumisawa *et al*., 2021). All the test subjects were prepared in triplicate.

Broth macrodilution method

The broth macrodilution assay was performed with slight modifications. Briefly, 9 mL of fresh nutrient broth was added into five sterile capped tubes respectively. Then, 1 mL of the *A. paniculata* DES-based extract was added to the first tube. The solution was mixed. Next, 1 mL of the mixture from the first tube was added to the second tube. This 10-fold serial dilution was continued until the fifth tube. The control medium (no inoculum & no extract) was also prepared. The *A. paniculata* DES-based extract was prepared in five diluted extracts from 2, 0.2, 0.02, 0.002 to 0.0002 mg/mL (w/v). Each tube of test solution consists of a combination of bacterial suspension (2 mL) and diluted extract (2 mL). The incubation was performed at 37°C for 24 hr. The lowest concentration of each tube that prevented visible growth was considered the minimal inhibitory concentration (MIC) (Nahr *et al*., 2018).

Antioxidant testing for *A. paniculata* DES based-extract

The antioxidant was determined by the DPPH radical-scavenging assay with slight modifications. The reaction mixture consisted of 3 mL DPPH (0.1 mM) combined with 1 mL of the extract. Ascorbic acid was used as the standard sample. The solution mixture was shaken and then placed in the dark for 30 min at room temperature. The absorbance was recorded using a spectrophotometer at 517 nm wavelength. Free radical scavenging activity (RSA) % was expressed as the inhibition percentage using Equation 1.

Equation 1:

$$\text{RSA } (\%) = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where:
$A_{control}$ = absorbance of DPPH solution without sample
A_{sample} = absorbance of test solution.

The IC_{50} value was plotted on a graph (inhibition % versus plant extracts) in a linear regression analysis to indicate antioxidant concentration where the sample is required to reduce the DPPH radical inhibition by 50% (Adamu *et al*., 2022).

Anti-inflammation testing for *A. paniculata* DES based-extract

The anti-inflammation was determined by the egg albumin denaturation assay with slight modifications. The source of albumin was from chicken egg white. The positive control was diclofenac sodium and the negative control was deionized water. The extract test solution was prepared by mixing egg albumin (0.2 mL), phosphate saline buffer with a pH of 6.4 (1.4 mL), and the extract (1 mL) in respective screw-capped tubes. The positive and negative controls were prepared in the same procedure as the extract. All the test solutions were incubated at 37°C for 15 min. Then, the solutions were heated for 5 min at 70°C and left to cool down at room temperature. The absorbance values for each solution were measured at the wavelength of 660 nm. The percentage inhibition of protein (albumin) denaturation was calculated based on Equation 2 (Sharmin *et al*., 2021):

Equation 2:

$$\text{Protein inhibition} = [V_c - V_t]/V_c \times 100\%$$

where:
V_t = Absorbance of the test sample
V_c = Absorbance of control

RESULTS AND DISCUSSION

Comparative analysis of deep eutectic solvent (DES) method about conventional methods

DES is a new promising solvent applicable in various applications, including the extraction of bioactive compounds. Generally, the output of different bioactive components varies according to the different parameters used (solvent & type of extraction). The drawback of conventional methods like Soxhlet, maceration, and soaking that use organic solvents (methanol, hexane, and petroleum ether) as solvent extractors could cause waste disposal problems due to the toxicity, thermal instability, and large volume of solvents required (Dheyab *et al*., 2021). The methods involved in the extraction and separation phase require laborious work. The extraction process would consume long periods: Soxhlet (6-48 hr), maceration (~3 days), and soaking (~24 hr), respectively (Raynie, 2019 ; Charu *et al*., 2022; Kholifah, *et al*., 2024). Whilst the separation phase commonly involved the use of a rotary evaporator required additional hr. Hence, this study focused on the potential of an eco-friendly DES as a solvent extractor while emphasizing the simple and rapid procedure extraction method, that has yet to be studied extensively.

This present method differs from previous conventional methods that often entail the generation of crude extract via rotary evaporation. The supernatant was utilized following the methodology established in previous research (Dabetić *et al*., 2020; Shikov *et al*., 2020; Wojeicchowski *et al*., 2021; Wu *et al*., 2021). It is recommended that the supernatant, which contains DES and extracted chemicals, can be employed directly without the need for solvent removal, especially if the use of DES has increased the biological activity.

The present DES is a blend of two elements (citric acid monohydrate & glycerol) that creates a eutectic combination with a melting point lower than that of any individual component. One of the primary benefits of eutectic solvents is their ability to create strong bonds, including hydrogen bonds, electrostatic forces, and *van der Waals* interactions (Calvo-Flores & Mingorance-Sánchez, 2021). When employed for extraction purposes, DES has a high degree of efficacy in the solubilization of various compounds from plant material (Hikmawanti *et al*., 2021). According to Cao *et al*. (2020), high solubility is related to DES-phytochemical interactions, such as dipole-dipole and hydrogen bonding interactions. The biocompatibility, flexibility, and high dissolving nature of DES allow it to be an idealistic delivery mechanism for bioactive compounds that do not require separation. Thus, these compounds are extracted with the bonds within the solvent along with an external factor applied through the centrifugal force during extraction. Moreover, the eutectic solvent itself is environmentally safe and positively contributes to the overall outcome. Therefore, the synergistic effect of the bioactive compounds and eutectic solvent is regarded as enhancing the overall quality of the bioactivity.

Synthesis of DES from the combination of citric acid monohydrate and glycerol

This study utilized citric acid monohydrate as the HBA and glycerol as the HBD. Due to their capacity for hydrogen bonding, both components have excellent solubility and tuneable viscosity. Citric acid monohydrate is a tricarboxylic organic acid, that provides various functionality because of its three carboxyl groups and one hydroxyl group (Lambros *et al*., 2022). Monohydrate is the inclusion of one water molecule per molecule of citric acid. Contrarily, glycerol is a trihydric alcohol with three hydroxyl groups joined to a carbon chain as shown in Figure 1 (Saraswat & Sengwa, 2023). Theoretically, these functional groups essentially facilitate strong hydrogen bonding interactions, crucial for eutectic synthesis (Koigerova *et al*., 2023). In addition, organic acids with carboxylic groups have a natural inclination to form hydrogen bonds, resulting in hydrophilic molecules that improve solubility in solvents (Hikmawanti *et al*., 2021; Swebocki *et al*., 2023). The inclusion of monohydrate, which is a naturally occurring water molecule can have an impact on viscosity, solubility, and bonding properties. The DES was synthesized with meticulous attention in regulated conditions by having consistent heating and stirring to guarantee the homogeneity and proper formation of the desired chemical bonds. A molar ratio of 1:2 had been tested and employed for the synthesis of DES. The DES was favorably synthesized as the mixture was stable and viscous at room temperature after 24 hr. Both components are environmentally sustainable and economically feasible, as they are inexpensive, readily accessible, and biodegradable. The versatility of these components allows for tailored properties, enhancing their suitability for specific extraction and beneficial in green chemistry applications.

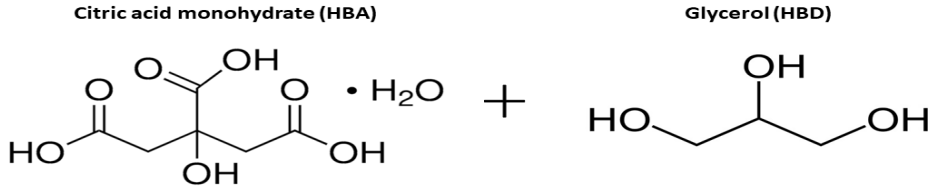

Fig. 1. The chemical structure of HBA and HBD.

Phytochemical screening

This method included the suspension of *A. paniculata* into the eutectic solvent, thereby effectively extracting the bioactive compounds. The DES solubility essentially catalyzes a more comprehensive extraction of target compounds. The phytochemical compounds were screened in *A. paniculata*-DES extract as presented in Table 1. Based on the tests, the plant extract exhibited the presence of steroids and terpenoids while tannins and alkaloids were absent.

Table 1. The phytochemical results of DES

Solvent	CA/Gly (flower & leaf extracts)
Tannins	X
Alkaloid	X
Terpenoid	/
Steroid	/

[#] X – not present
/ – present

Different technical adjustments need to be considered during extraction such as the soaking time, centrifugation conditions as well as the characterization of selected DES as it may affect the yield and type of metabolites within the plants (Suthar *et al*., 2023). DESs are mixtures that possess a significantly lower eutectic point making them stable and idealistic at ambient temperatures (Serna-Vázquez *et al*., 2021). Thus, it can be easily applied in products in the future. Steroids are part of lipid groups therefore they tend to dissolve in non-polar solvents. However, steroids can also be extracted in polar and semi-polar solvents like ethanol and ethyl acetate (Wutsqa *et al*., 2021; Mulyani & Si, 2022). Steroids were present in this study because DES have multiple interactions such as hydrogen bonding, dipole-dipole, dipole-induced dipole, *van der Waals* (dispersion), and ion-dipole interactions that allow extraction of polar and non-polar components (Khataei *et al*., 2018). Next, terpenoid compounds also can be attracted to semipolar or even polar solvents as they possess a hydroxyl group substituent attached to the hydrocarbon chain (Wutsqa *et al*., 2021). The results found that both steroids and terpenoids were present in flower and leaf extracts of *A. paniculata*. The absence of tannins and alkaloids may be related to the efficiency and characterization of DES to attract these compounds as the DES used is relatively new and still under investigation.

Generally, the extraction of solute and the solvents are affected by polarity, chemical structure, extraction concentration, vapor pressure, and acid-base properties (Herawati & Pudjiastuti, 2021). DES presents remarkable flexibility as the alteration of DES characteristics such as the molar ratio of the individual components, temperature, and water content can affect the physico-chemical properties (fluidity, conductivity & polarity) (El Achkar *et al*., 2019; Gabriele *et al*., 2019). DES comprises a mixture of citric acid monohydrate and glycerol which is more natural and environmentally friendly. DES is highly adaptable as the individual components involved can be ionic or non-ionic, polar or non-polar. Thus, the versatility of DES can dissolve a wider range of compounds compared to organic solvents (Liu *et al*., 2018).

Antibacterial activities of DES-leaf and DES-flower extracts

Acmella paniculata has multiple bioactive compounds that can provide antibacterial properties against pathogenic microbes. In this study, the antibacterial test was used to determine the bacterial

response towards both extracts (leaf & flower). Table 2 displays the inhibition zone of all the test subjects. Based on the analysis, all the tested bacteria were susceptible to the flower and leaf extracts. According to a previous study by Thakur *et al*. *(*2019), the strong antibacterial activity of the plant is due to the presence of highly crucial bioactive compounds such as phenolics, flavonoids, coumarins, and triterpenoids. The bacterial strains were found to be more susceptible to the DES-flower extract. The highest inhibition zone (21.33±0.58 mm) was shown by 60% DES-flower extract against *S. aureus* which determines the susceptibility response of the tested pathogen. Besides, in the activity found within DES-leaf extracts, *B. subtilis* exhibited a susceptible response with the largest inhibition zone (18.33±0.58 mm) observed at 70% DES-leaf extract. Consequently, the antibacterial efficacy of *A. paniculata* flower extract is superior in comparison to the leaf part. It was also exhibited that the different concentrations of DES had contributed to the bacterial responses and different inhibition zones towards the strains.

Table 2. Antibacterial activity of plant extracts in different concentrations

Parts	Sample	Diameter of inhibition zone (mm)			
		Gram-negative		Gram-positive	
		E. coli ATCC	*Salmonella enterica* ser. Typhimurium	*B. subtilis* ATCC	*S. aureus* ATCC
Leaf	70% DES	17.67±1.15**	15.67±0.58**	**18.33±0.58****	16.00±1.00**
	60% DES	17.33±0.58	15.33±0.58	**17.67±1.53**	15.33±0.58**
	50% DES	15.67±1.15	14.00±1.00	**16.67±1.53**	16.33±1.15**
Flower	70% DES	**17.33±0.58****	**16.67±0.58****	18.00±1.00**	**19.67±1.15****
	60% DES	**17.67±1.15**	**15.00±1.00**	16.67±1.53	**21.33±0.58****
	50% DES	**16.00±1.00**	**13.67±1.53**	15.67±1.15	**20.67±0.58****
DES only	70% DES	21.33±1.53**	21.33±0.58**	21.67±1.53**	18.33±0.58**
	60% DES	18.00±1.00	22.00**±**1.00	19.00±1.00	20.67±1.15**
	50% DES	16.67±1.15	20.67**±**1.15	18.33±0.58	18.67±0.58**
Positive control	Gentamicin (10 µg/ disc)	18.33±1.53	17.67±0.58	18.33±1.53	19.67±0.58

[#] Note – The data were expressed as the mean value with standard deviation. **Indicates the significant difference between values (p<0.05), according to one-way ANOVA

On the other hand, it could be suggested that DES itself may have heavily influenced the antibacterial bioactivity against all bacteria. Approximately, 60% DES recorded the largest inhibition zone 22.00±1.00 mm against *Salmonella enterica* ser. Typhimurium. DES presented varying antibacterial properties based on the types of composition and concentration used (Bedair *et al*., 2024). The antibacterial properties in DES itself can further optimize the synergistic effects of compounds. According to Jurić *et al*. (2021), DES with acidic components was proven to have promising antibacterial activities against *S. aureus, E. coli,* and *Salmonella enterica* ser. Typhimurium. The high antibacterial activity of DES with organic acids was because of the low pH (lower than 3) making it capable of inhibiting bacterial growth development as the optimum microbial growth is typically 6.5 to 7.5. The acidic surrounding increases the toxicity towards the microbial cells, therefore disrupting the membrane and causing protein denaturation. Thus, the outcome indicated that DES could potentially become an enhancer for the plant extracts in antibacterial activities (Bedair *et al*., 2024).

The minimal inhibitory effect was tested on only the highest antibacterial activity for every bacterium based on the results from the disc diffusion method. The effects of various DES-plant extracts, specifically 60% DES-flower, 70% DES-flower, 70% DES-leaf, and 60% DES-leaf, were evaluated against *E. coli*, *Salmonella enterica* ser. Typhimurium, *B. subtilis*, and *S. aureus*. Generally, all the selected extracts showed equal minimal inhibitory effects. After incubation, test tubes 2 mg/mL and 0.2 mg/mL showed no visible turbidity indicating no bacterial growth, whereas test tubes 0.02 mg/mL, 0.002 mg/mL, and 0.0002 mg/mL had visible turbidity, exhibiting bacterial growth. This classifies the MIC value at 0.2 mg/mL (w/v), which was the second serial dilution. Hence, *A. paniculata* could become a potential antibacterial agent to inhibit the growth of particular pathogens. Theoretically, antibacterial activity is involved when the agent can weaken or lysis the microbe cell wall membranous tissue (Tavares *et al*., 2020). MIC values that are above 1 mg/mL indicate inactive activity while MIC values below 0.1 mg/mL indicate promising antibacterial activities (Charu *et al*., 2022). Therefore, the antibacterial activities against four bacterial strains could be considered moderate but promising due to the significant disc diffusion results for further analysis in natural products.

Antioxidant activities of DES-leaf and DES-flower extracts

Free radical scavengers are antioxidant substances that stabilize and protect cells from damage caused by free radicals (Chaudhary *et al*., 2023). Overall, DES flower extract showed higher radical scavenging activity (RSA) ranging from 75%-77% compared to the leaf extract which ranged from 65%-69%. The highest antioxidant activity for both leaf and flower extracts in 70% DES were 69.73±0.76% and 77.49±0.90% respectively. It was also observed that the RSA for both extracts increased as the DES concentration increased. Moreover, DES itself showed an RSA of 55.95±0.97%, 56.37±1.07%, and 55.25±0.41% for 70%, 60%, and 50% (v/v) accordingly indicating a small contribution to the overall antioxidant activity. Figures 2, 3, and 4 show the comparison of antioxidant activity for *A. paniculata* based on different concentrations and plant parts. The IC_{50} indicates the concentration of an antioxidant substance needed to efficiently scavenge initial DPPH radicals by 50%. The data presented in Table 3 indicates the IC_{50} value for DES-leaf with DES-flower extracts giving a value of 10.08 µg/mL and 25.61 µg/mL respectively, while for DES alone was 18.90 µg/mL. The IC_{50} detected was lower than the ascorbic acid standard of 29.92 µg/mL. A lower IC_{50} value stipulates a more effective extract in scavenging DPPH, leading to higher antioxidant activity (Ramadan *et al*., 2022). According to Al Ragib *et al*. (2020), *A. paniculata* flowers result in promising antioxidant capacity in extraction solvents that vary in polarities such as water, chloroform, ethanol, and n-hexane. Moreover, a similar study by Thakur *et al*. *(*2019) showed high antioxidant activity in both the leaf and flower of *A. paniculata* extracts with methanol, acetone, and water as the solvent extractor. Previous literature states that the promising antioxidant activity of *A. paniculata* is commonly caused by the presence of bioactive compounds such as phenolics (phenolic acids & carnosic acid), flavonoids (epigallocatechin-3-gallate & quercetin) and steroids (Kelsey *et al*., 2010; Andriani *et al*., 2019; Rahim *et al*., 2021). The phenolic compound can chelate metal ions during the production of free radicals.

Table 3. Antioxidant activity of plant extracts in different concentrations

	Samples (DES-Water Percentage)	RSA (%)	IC_{50}
	DES		µg/mL
Leaf	70% DES	69.73±0.76**	10.08
	60% DES	66.23±0.90**	
	50% DES	65.49±0.76**	
Flower	70% DES	77.49±0.90**	25.61
	60% DES	76.96±0.79**	
	50% DES	75.24±1.05**	
DES only	70% DES	55.95±0.97**	18.90
	60% DES	56.37±1.07**	
	50% DES	55.25±0.41**	

Note – The data were expressed as the mean value with standard deviation. **Indicates the significant difference between values (p<0.05), according to one-way ANOVA

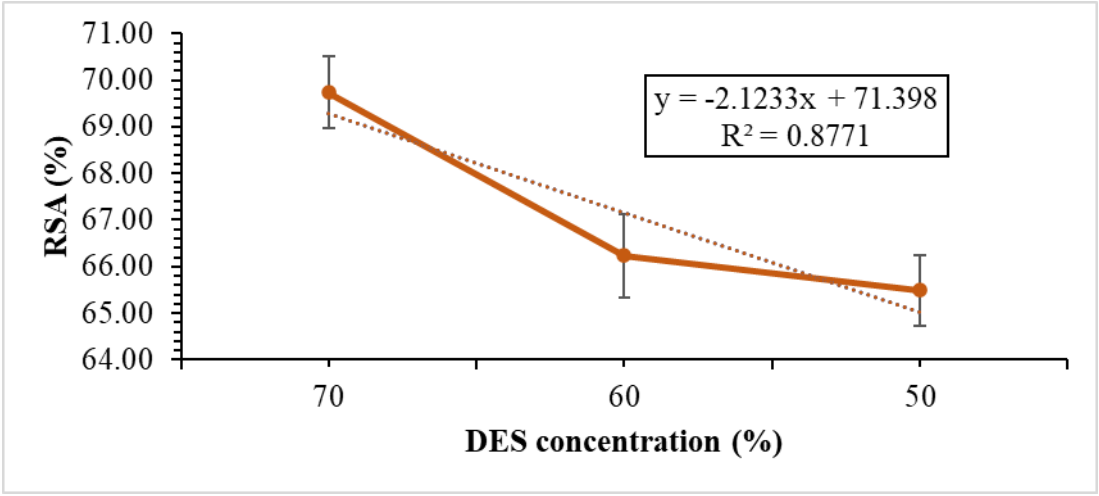

Fig. 2. Radical scavenging assay (%) for leaf *A. paniculata* extract.

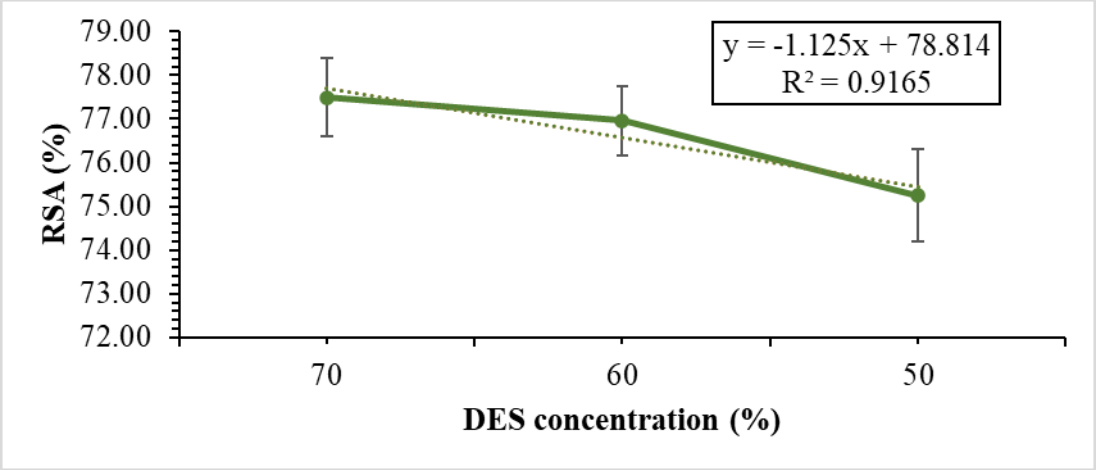

Fig. 3. Radical scavenging assay (%) for flower *A. paniculata* extract.

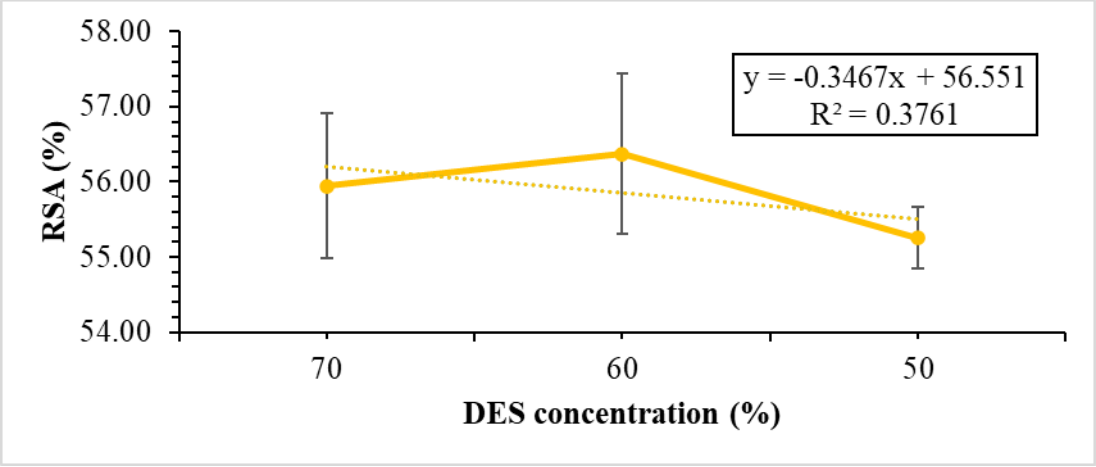

Fig. 4. Radical scavenging assay (%) for only DES.

Anti-inflammatory activities of DES-leaf and DES-flower extracts

The most common cause of autoantigen production in inflammation is the denaturation of proteins. This method identifies the ability of an extract to stop protein from denaturation under extreme circumstances such as excessive stress, heat, pH, or other external agents (Yesmin *et al*., 2020). Physical denaturation is when the egg albumin disrupts its tertiary and secondary structure. This leads to the protein losing its biological functions which commonly leads to inflammation or tissue damage. Therefore, drugs or extracts with anti-inflammatory properties should theoretically be able to stabilize protein structures by avoiding or reducing protein denaturation (Madhuranga & Samarakoon, 2023). The decrease in absorbance translates to the inhibition of protein (albumin) denaturation (Mirke *et al*., 2020). As presented in Table 4, the DES-flower extracts indicated greater inhibition of protein denaturation rates of 95.21%, 91.59%, and 93.29% at different DES concentrations in extracts. Whereas, the DES-leaf extracts for all three DES concentrations showed slightly lower inhibition of protein denaturation rates of 82.11%, 89.46%, and 84.77% respectively. Hence, the albumin denaturation assay DES-flower extract had a better anti-inflammatory effect than the DES-leaf extract. The highest anti-inflammatory activity ascertained in DES-flower extract was prepared using 70% DES. Moreover, DES alone also showed anti-inflammatory properties contributing to the overall inhibition of protein denaturation rate. The albumin denaturation capacity for diclofenac sodium (standard) had only ranged from 23.44% to 75.08% for 2-10 mg/mL as indicated in Table 5. This extract showed higher anti-inflammatory activity as compared to the standard. Gairola *et al*. (2022), conducted a study based on albumin denaturation using a similar species known as *Acmella uliginosa* by using methanol and hexane as solvent extractors. The extracts of *A. uliginosa* presented higher results as compared to the diclofenac sodium standard validifying its anti-inflammation properties.

Table 4. Anti-inflammatory capacity of leaf and flower extracts

	Samples (DES-Water Percentage)	Albumin inhibition (%)
CA/Gly		
Leaf	70% DES	82.11±1.47**
	60% DES	89.46±0.85**
	50% DES	84.77±1.02**
Flower	70% DES	95.21±0.65**
	60% DES	91.59±1.51**
	50% DES	93.29±1.15**
DES only	70% DES	61.45±1.12**
	60% DES	60.81±0.98**
	50% DES	58.79±1.15**

Note – The data were expressed as the mean value with standard deviation. **Indicates the significant difference between values (p<0.05), according to one-way ANOVA

Table 5. Anti-inflammatory capacity of diclofenac sodium

Diclofenac sodium (mg/mL)	Albumin inhibition (%)
2	23.44±1.03**
4	34.19±1.17**
6	56.82±1.95**
8	68.37±0.87**
10	75.08±1.91**

Note – The data were expressed as the mean value with standard deviation. **Indicates the significant difference between values (p<0.05), according to one-way ANOVA

Statistical analysis

As for the antibacterial test, the 70% DES concentration was significant for all the pathogens excluding *S. aureus* which was significant for all concentrations. Next, both antioxidant and anti-inflammation tests also presented statistical significance for DES concentrations.

CONCLUSION

The composition of citric acid monohydrate (HBA) with glycerol (HBD) was successfully formed into a new eutectic liquid (DES) at room temperature. Therefore, it reflects chemical stability characteristics suitable for high temperatures or harsh chemical-related processes such as the separation and purification of bioactive compounds. Subsequently, DES exhibited tremendous potential due to its adaptability, solubility, and tuneable characteristics for an environmentally friendly effective solvent extractor. Overall, the DES-flower extract showed higher bioactivities when compared to the DES-leaf extract. It was also observed that the different concentrations of DES affected the bioactivity outcome. The positive bioactivities may be due to the bioactive constituents found in the extracts. This extract therefore can become a new promising active ingredient in topical products such as in open wound remedies.

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ETHICAL STATEMENT

Not applicable

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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