BIOLOGY, MEDICINE, & NATURAL PRODUCT CHEMISTRY ISSN 2089-6514 (paper)
Volume 15, Number 2, October 2026 | Pages: 2025-2031 | DOI: 10.14421/biomedich.2026.152.2025-2031 ISSN 2540-9328 (online)

Comparative Evaluation of Ethanol and Malic Acid–Glycerol Deep Eutectic Solvent for the Extraction of Antioxidant Phytochemicals from *Casuarina equisetifolia* Stem Bark

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Manuscript received: 03 August 2026. Revision accepted: 03 November 2026, Published: 07 November 2026.

Abstract

The growing interest in sustainable extraction technologies has promoted the application of deep eutectic solvents (DESs) as environmentally friendly alternatives to conventional organic solvents for recovering bioactive compounds from plant materials. However, comparative studies between ethanol and DES for extracting antioxidant compounds from *Casuarina equisetifolia* stem bark remain limited. This study evaluated the antioxidant potential of *C. equisetifolia* stem bark extracts obtained using a malic acid–glycerol DES and 96% ethanol. The DES was synthesized at a glycerol-to-malic acid molar ratio of 2:1 and characterized by Fourier transform infrared (FTIR) spectroscopy. Stem bark extraction was performed using ultrasound-assisted extraction (UAE), followed by qualitative phytochemical screening, determination of total phenolic content (TPC) using the Folin–Ciocalteu method, and antioxidant activity evaluation using the DPPH radical-scavenging assay. FTIR analysis confirmed the successful formation of the DES through characteristic shifts in the O–H and C=O stretching bands, indicating hydrogen-bond interactions between glycerol and malic acid. Qualitative phytochemical screening revealed the presence of phenolics, flavonoids, terpenoids, and saponins in both extracts. The DES extract exhibited a higher total phenolic content (50.3 mg GAE g^{-1} extract) than the ethanol extract (42.2 mg GAE g^{-1} extract). Consistently, the DES extract demonstrated stronger antioxidant activity with an IC_{50} value of 42.2 ppm, compared with 54.6 ppm for the ethanol extract. The enhanced extraction performance of the DES is attributed to its hydrogen-bonding network, which facilitates the recovery of phenolic antioxidants. These findings demonstrate that the malic acid–glycerol DES is an effective green extraction solvent for producing antioxidant-rich extracts from *C. equisetifolia* stem bark and supports its potential application in the development of sustainable cosmetic ingredients.

Keywords: *Casuarina equisetifolia*; deep eutectic solvent; ultrasound-assisted extraction; antioxidant activity; total phenolic content.

INTRODUCTION

The increasing demand for naturally derived and sustainable cosmetic ingredients has stimulated extensive research into plant-based sources of bioactive compounds. Natural antioxidants are particularly attractive because oxidative stress and the excessive formation of reactive oxygen species (ROS) are closely associated with cellular damage and skin aging (Hussen et al., 2025). The accumulation of oxidative damage can contribute to collagen degradation, inflammation, pigmentation, and other processes associated with premature skin aging (Lee et al., 2021). Consequently, botanical extracts containing antioxidant compounds have become important candidates for the development of cosmetic ingredients with potential protective and anti-aging functions.

Phenolic compounds and flavonoids are among the most important secondary metabolites associated with antioxidant activity in plants. Their antioxidant properties are primarily related to their ability to donate hydrogen atoms or electrons to reactive species and stabilize free radicals (Platzer et al., 2022). In addition, tannins and other phenolic derivatives may contribute to the overall radical-scavenging capacity of plant extracts. The total phenolic content (TPC) is therefore frequently used as an important chemical parameter when evaluating the antioxidant potential of botanical extracts. However, antioxidant activity is influenced by the overall phytochemical composition and the interactions among different compounds rather than by a single metabolite group.

Casuarina equisetifolia L. is a coastal tree widely distributed in tropical and subtropical regions. Although it is primarily recognized for its ecological function in

coastal protection, its biomass contains various secondary metabolites with potential biological activities. Previous research has demonstrated the presence of phenolic compounds and condensed tannins in *C. equisetifolia* needles extract, supporting its potential as a natural source of antioxidant compounds (K. & Gowrie, 2018). More recently, investigation of different *C. equisetifolia* extracts further demonstrated the presence of phenolic and flavonoid constituents and confirmed antioxidant activity (Abdallah et al., 2024), although the phytochemical composition and biological activity varied depending on the extraction solvent.

Ethanol is one of the most widely applied solvents for plant extraction because of its effectiveness in recovering polar and moderately polar compounds, relatively low toxicity, ease of removal, and established application in pharmaceutical, food, and cosmetic processing (Abdallah et al., 2024; K. & Gowrie, 2018). These characteristics make ethanol particularly relevant for the development of botanical cosmetic ingredients. Nevertheless, solvent selection remains an important factor because the polarity and physicochemical characteristics of the extraction medium influence the quantity and composition of recovered phytochemicals.

Deep eutectic solvents (DESs) have recently emerged as promising green extraction media because of their low volatility, biodegradability, and tunable physicochemical properties, which enable efficient recovery of bioactive compounds from plant materials (Carbonell-Rozas et al., 2024; Kamiloğlu et al., 2025; Lazović et al., 2022). Organic acid-based DESs, particularly those composed of malic acid and glycerol, have demonstrated enhanced extraction performance for phenolic compounds owing to their extensive hydrogen-bonding network and adjustable polarity (Koigerova et al., 2023). Nevertheless, despite these advantages, ethanol remains the solvent of choice in most cosmetic and pharmaceutical industries because of its well-established safety profile, regulatory acceptance, ease of solvent recovery, and compatibility with large-scale production processes. Consequently, comparing ethanol with an emerging green solvent such as DES is important not only to evaluate extraction efficiency but also to determine whether conventional ethanol extraction remains suitable for producing antioxidant-rich botanical extracts intended for cosmetic applications.

Although the phytochemical composition and biological activities of *C. equisetifolia* have been reported in several studies, comprehensive information regarding the relationship between phytochemical constituents, total phenolic content, and antioxidant activity of the stem bark extracted with ethanol remains limited. Furthermore, studies directly comparing ethanol extraction with a malic acid–glycerol DES for *C. equisetifolia* stem bark are still scarce. Such a comparison provides valuable insight into the influence of extraction solvent on phytochemical recovery and antioxidant performance while highlighting the practicality of ethanol as an industrial extraction solvent.

Therefore, this study aimed to evaluate the antioxidant potential of *Casuarina equisetifolia* stem bark ethanol extract through qualitative phytochemical screening, determination of total phenolic content, and DPPH radical-scavenging activity. A malic acid–glycerol deep eutectic solvent extract was included as a comparative extraction system to provide a benchmark for extraction performance and to examine the relationship between solvent selection, phytochemical composition, phenolic content, and antioxidant activity. The findings are expected to provide scientific evidence supporting the utilization of *C. equisetifolia* stem bark as a sustainable source of natural antioxidant ingredients while offering practical insights into the application of conventional and green extraction solvents for cosmetic product development.

MATERIALS AND METHODS

Materials

Stem bark of *Casuarina equisetifolia* was collected from Institut Teknologi Sumatera Botanical Garden. The plant material was cleaned to remove soil and other adhering materials and subsequently dried under room temperatutre. The dried stem bark was ground into powder and stored in a closed container until extraction. Other chemicals used are malic acid, gliserol and ethanol.

Procedures

Preparation of Malic Acid–Glycerol Deep Eutectic Solvent

The DES was prepared from glycerol and malic acid. Both components were mixed at the 2:1 molar ratio and heated under continuous stirring until a homogeneous liquid was obtained, indicating the formation of the eutectic system. The synthesized DES was allowed to cool to room temperature and subsequently stored in a sealed container until use. The formation of the DES was confirmed by Fourier Transform Infrared (FTIR) spectroscopy, with spectra recorded over the range of 4000–400 cm^{-1}. The FTIR analysis was performed to verify hydrogen-bond interactions between malic acid and glycerol through the characteristic shifts and broadening of functional group absorption bands compared with those of the individual precursor compounds. After characterization, the synthesized DES was used as the extraction solvent for Casuarina equisetifolia stem bark

Extraction of C. equisetifolia stem bark

The powdered stem bark of *Casuarina equisetifolia* L. was extracted using the Ultrasound-Assisted Extraction (UAE) method with the synthesized deep eutectic solvent (DES) as the primary extraction solvent, while ethanol

96% was used as the conventional extraction solvent for comparison. The extraction was performed at a solid-to-solvent ratio of 1:10 (w/v).

For each extraction, the powdered sample was mixed with the respective solvent in a suitable extraction vessel and subjected to ultrasonic treatment using an ultrasonic bath operating at a frequency of 40 kHz. Extraction was carried out at two extraction temperatures (40°C and 50°C) for 30 min under identical operating conditions. Upon completion of the extraction process, the extracts were filtered to separate the liquid extract from the plant residues. The resulting filtrates were collected for subsequent phytochemical screening, total phenolic content determination, and antioxidant activity analysis.

Phytochemical Screening

Qualitative phytochemical screening was performed to identify the major classes of secondary metabolites present in the ethanol and deep eutectic solvent (DES) extracts of Casuarina equisetifolia stem bark. The screening was conducted using standard phytochemical procedures with slight modifications.

Phenolic Compounds

The presence of phenolic compounds was determined using the ferric chloride ($FeCl_3$) test. 1 mL of each extract was mixed with a few drops of 5% ferric chloride solution. The appearance of a dark blue, bluish-black, or greenish-black coloration indicated a positive result for phenolic compounds.

Flavonoids

Flavonoids were identified using the Shinoda test. Briefly, 1 mL of extract was mixed with a small amount of magnesium ribbon, followed by the addition of several drops of concentrated hydrochloric acid (HCl). The development of an orange, pink, or reddish coloration indicated the presence of flavonoids.

Terpenoids

The presence of terpenoids was evaluated using the Salkowski test. 1 mL of extract was mixed with chloroform, followed by careful addition of concentrated H_2SO_4 along the side of the test tube to form a separate layer. The formation of a reddish-brown interface indicated a positive reaction for terpenoids.

Saponins

Saponins were detected using the frothing test. 1 mL of extract was diluted with distilled water and vigorously shaken for 30 s. The formation of a stable foam layer persisting for at least 10 min indicated the presence of saponins.

Alkaloids

Alkaloids were detected using Mayer's reagent. 1 mL of the extract was acidified with a few drops of dilute hydrochloric acid (HCl), followed by the addition of 2–3 drops of Mayer's reagent. The formation of a creamy white or yellowish-white precipitate indicated the presence of alkaloids.

Tannins

Tannins were identified by adding a few drops of 1% ferric chloride solution to the extract. The formation of a dark blue or greenish-black coloration indicated the presence of tannins.

All phytochemical tests were performed in triplicate, and the results were recorded qualitatively as present (+) or absent (−) based on the appearance of characteristic color changes, precipitates, or stable foam formation.

Table 1. Qualitative phytochemical tests used for C. equisetifolia stem bark extracts.

Phytochemical	Reagent	Positive Indication
Phenolic	5% FeCl3	Blue-black or greenish-black color
Flavonoid	Mg ribbon + conc. HCl (Shinoda)	Orange/red/pink color
Terpenoid	Chloroform + conc. H_2SO_4 (Salkowski)	Reddish-brown interface
Saponin	Distilled water (frothing test)	Stable foam (>10 min)
Alkaloid	Mayer's reagent	White precipitate
Tannin	1% $FeCl_3$	Blue-black or greenish-black color

DPPH radical scavenging activity

The antioxidant activity of the extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Different concentrations of each extract were reacted with DPPH solution and incubated under controlled conditions. The absorbance was subsequently measured using a UV-Vis spectrophotometer at the appropriate wavelength.

The percentage of DPPH radical inhibition was calculated using:

$$\text{Inhibition (\%)} = \frac{A_0 - A_s}{A_0} \times 100$$

where $A_0$is the absorbance of the DPPH control and A_s is the absorbance of the sample.

The inhibition percentage was plotted against extract concentration, and linear regression was used to estimate the concentration required to inhibit 50% of DPPH radicals (IC_{50}).

Determination of Total Phenolic Content

Total phenolic content (TPC) was determined using the Folin–Ciocalteu colorimetric method. Gallic acid was used as the reference standard. A calibration curve was prepared from a series of gallic acid concentrations, and absorbance was measured at the appropriate wavelength.

The total phenolic content of each extract was calculated from the gallic acid calibration curve and expressed as mg gallic acid equivalent (GAE)/g extract.

Data analysis

The DPPH antioxidant activity was evaluated based on the IC_{50} value obtained from the linear regression equation. The phytochemical screening results were interpreted qualitatively, while TPC was expressed as gallic acid equivalents. Comparative interpretation was performed between ethanol and DES extracts to determine the relationship between phytochemical composition, phenolic content, and antioxidant activity.

RESULTS AND DISCUSSION

Results

Infra-red Spectra

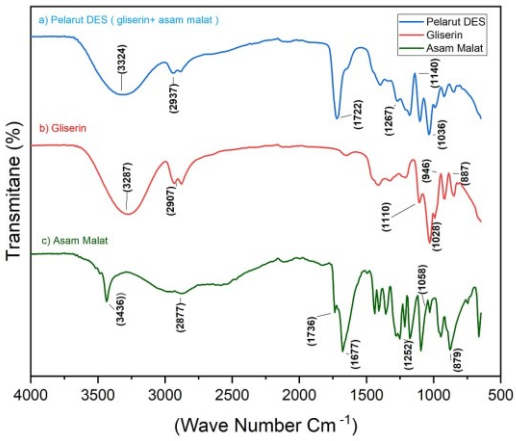


Figure 1 Infrared spectra of gliserin, malic acid and DES.

Phytochemical Screening

Table 2. Phytochemical screening of casuarina extract.

Phytochemical profile	Sample Casuarina extract ethanol	Casuarina extract DES
Phenolic	+	+
Flavonoid	+	+
Terpenoid	+	+
Saponin	+	+
Alkaloid	+	+
Tannins	+	+

Antioxidant activity of Casuarina Extract

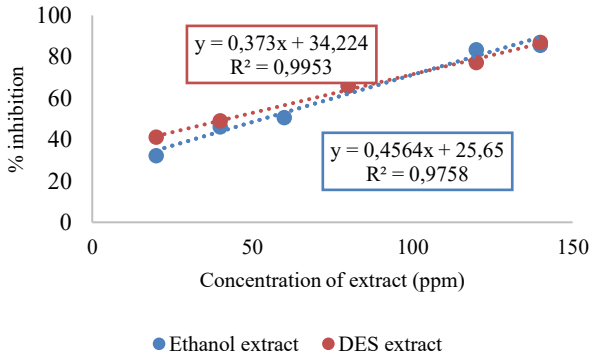


Figure 2 Linear regresion of Casuarina extract concentration versus inhibition % of radical scavenging

Table 3 IC_{50} of extract Casuarina.

Sample	IC50 (ppm)
Casuarina Extract DES	42.2
Casuarina Extract Ethanol	53.4

Total Phenolic Content of Casuarina Extract

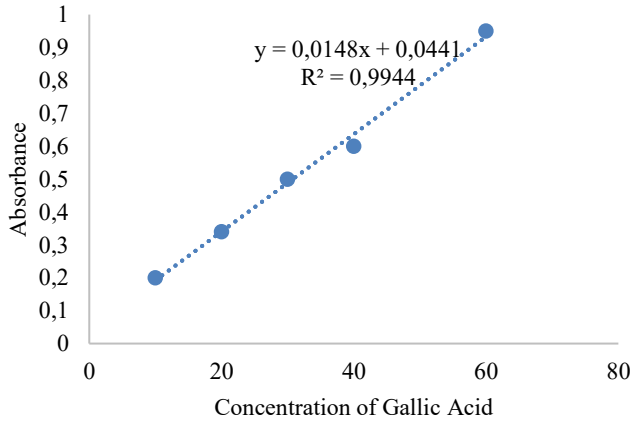


Figure 3. Standard curve of gallic acid.

Table 4. Total phenolic content of Casuarina extracts.

Sample	Absorbance	Total Phenolic Content (mg/GAE)
DES extract	0.7883	50.3
Ethanol extract	0.6693	42.2

Discussion

The antioxidant potential of *C. equisetifolia* stem bark extracts was comprehensively evaluated through a combination of solvent characterization, qualitative phytochemical analysis, total phenolic content determination, and DPPH radical-scavenging assay. Since the extraction performance of a solvent is closely related to its physicochemical properties, the synthesized malic acid–glycerol deep eutectic solvent (DES) was first characterized using Fourier Transform Infrared (FTIR)

spectroscopy to verify the formation of the eutectic system.

The successful formation of the malic acid–glycerol deep eutectic solvent (DES) was verified by Fourier Transform Infrared (FTIR) spectroscopy through comparison of the spectra of the synthesized DES with those of its individual precursor components, glycerol and malic acid (Figure 1). The FTIR spectrum of glycerol exhibited a broad absorption band at 3287 cm^{-1}, corresponding to O–H stretching vibrations, together with C–H stretching at 2907 cm^{-1} and characteristic C–O stretching bands at 1110, 1028, 946, and 887 cm^{-1}. Malic acid displayed a broad O–H stretching band at 3436 cm^{-1}, a strong carbonyl (C=O) stretching vibration at 1736 cm^{-1}, and characteristic absorptions at 1677, 1252, 1058, and 879 cm^{-1}, which are attributed to C–O stretching and O–H deformation of carboxylic acid groups.

After synthesis, the DES spectrum exhibited noticeable shifts in several characteristic absorption bands. The broad O–H stretching vibration appeared at 3324 cm^{-1}, located between those of glycerol and malic acid, while the C–H stretching band shifted to 2937 cm^{-1}. The carbonyl stretching vibration also shifted from 1736 cm^{-1} in pure malic acid to 1722 cm^{-1} in the DES. Furthermore, absorption bands at 1267, 1140, and 1036 cm^{-1} became more prominent after DES formation.

These spectral changes indicate extensive intermolecular hydrogen bonding between the hydroxyl groups of glycerol and the carboxyl groups of malic acid. The shift of the O–H stretching vibration toward lower wavenumbers and the displacement of the carbonyl stretching band are characteristic signatures of hydrogen-bond formation in deep eutectic solvents (Shishov et al., 2022). No additional absorption peaks corresponding to new covalent bonds were observed, confirming that DES formation occurred through non-covalent interactions rather than chemical reactions (Kitamura & Torii, 2022).

The formation of this hydrogen-bonded network is expected to modify the physicochemical characteristics of the solvent, including polarity, viscosity, and solvation capacity. These changes are particularly important because they influence the extraction selectivity toward polar phytochemicals such as phenolic compounds and flavonoids. Therefore, FTIR analysis provides structural evidence supporting the subsequent evaluation of the synthesized DES as an alternative extraction solvent for *C. equisetifolia* stem bark.

Following confirmation of DES formation by FTIR spectroscopy, qualitative phytochemical screening was performed to compare the classes of secondary metabolites extracted by ethanol and the synthesized malic acid–glycerol DES. The results are summarized in Table 1. Both extraction systems successfully recovered several major groups of secondary metabolites, including phenolic compounds, flavonoids, terpenoids, and saponins. No marked qualitative differences were observed between the ethanol and DES extracts, indicating that both solvents were capable of extracting the principal bioactive constituents present in the stem bark.

The detection of phenolic compounds and flavonoids is particularly important because these metabolites are widely recognized as the primary contributors to antioxidant activity. Their hydroxyl groups can donate hydrogen atoms or electrons to neutralize free radicals, thereby interrupting oxidative chain reactions (Platzer et al., 2022). Flavonoids further enhance antioxidant activity through resonance stabilization of phenoxyl radicals, whereas terpenoids and saponins may contribute indirectly through complementary antioxidant and anti-inflammatory mechanisms.

Although both extraction systems yielded similar qualitative phytochemical profiles, qualitative screening only indicates the presence or absence of metabolite groups and does not provide information regarding their abundance or chemical composition. Therefore, differences in antioxidant activity observed between the extracts cannot be explained solely by phytochemical screening and require further evaluation through quantitative analyses.

Interestingly, the FTIR characterization suggests that the hydrogen-bond network established within the DES may alter solvent polarity and molecular interactions with plant metabolites. Such interactions may influence extraction selectivity, enabling the recovery of different proportions of antioxidant constituents despite producing similar qualitative phytochemical profiles.

The antioxidant activities of the extracts were evaluated using the DPPH radical scavenging assay, and the results are presented in **Table 2**. The ethanol extract exhibited an IC_{50} value of 54.6 ppm, whereas the DES extract showed a lower IC_{50} value of 42.2 ppm, indicating stronger radical-scavenging activity. Both extracts demonstrated considerable antioxidant capacity, suggesting that *C. equisetifolia* stem bark is a promising source of natural antioxidant compounds. Nevertheless, the lower IC_{50} value obtained for the DES extract indicates that the synthesized solvent recovered antioxidant constituents with greater radical-scavenging efficiency than those extracted by ethanol.

This difference may be explained by the physicochemical properties of the DES demonstrated by FTIR analysis. The extensive hydrogen-bond interactions formed between glycerol and malic acid modify solvent polarity and intermolecular interactions, thereby improving the solubilization of specific phenolic compounds. Rather than extracting a larger quantity of phenolics, the DES may preferentially dissolve compounds possessing stronger antioxidant activity.

The antioxidant mechanism is mainly associated with the ability of phenolic hydroxyl groups to donate hydrogen atoms to the stable DPPH radical. The resulting phenoxyl radicals are stabilized through resonance, reducing the propagation of oxidative reactions. Consequently, the antioxidant performance depends not

only on the total concentration of phenolics but also on the molecular structures and substitution patterns of the extracted compounds.

Although the DES extract demonstrated superior antioxidant activity, the ethanol extract remains highly relevant for industrial applications. Ethanol is widely accepted in cosmetic and pharmaceutical manufacturing because of its safety, regulatory acceptance, low toxicity, and ease of solvent recovery. Therefore, the IC_{50} value of 54.6 ppm still demonstrates that ethanol extraction effectively recovers antioxidant compounds suitable for cosmetic ingredient development.

The total phenolic content (TPC) of the extracts was determined using the Folin–Ciocalteu assay to evaluate the contribution of phenolic compounds to antioxidant activity. The results are presented in Table 3. The malic acid–glycerol DES extract exhibited a higher total phenolic content (50.3 mg GAE g^{-1} extract) than the ethanol extract (42.2 mg GAE g^{-1} extract). This result indicates that the synthesized DES was more effective in extracting phenolic compounds from C. equisetifolia stem bark than the conventional ethanol solvent.

The higher TPC observed in the DES extract can be attributed to the unique physicochemical characteristics of deep eutectic solvents. FTIR characterization confirmed the successful formation of an extensive hydrogen-bond network between malic acid and glycerol, which may enhance solvent polarity and improve interactions with hydroxyl-rich phytochemicals. These hydrogen-bonding interactions facilitate the dissolution of phenolic compounds, thereby increasing their extraction efficiency. Phenolic compounds are among the major contributors to antioxidant activity because their hydroxyl groups readily donate hydrogen atoms or electrons to neutralize reactive oxygen species and stabilize free radicals. Consequently, extracts containing higher concentrations of phenolic compounds generally exhibit stronger radical-scavenging activity.

One of the most important findings of this study is the consistency observed between the structural characteristics of the synthesized DES, the recovery of phenolic compounds, and the antioxidant activity of the resulting extracts. FTIR analysis confirmed the successful formation of the malic acid–glycerol DES through intermolecular hydrogen bonding, as evidenced by the shifts in the O–H and C=O stretching vibrations. The establishment of this hydrogen-bonded network modifies the physicochemical properties of the solvent, including its polarity and solvation capacity, which are important factors governing the extraction of polar phytochemicals.

Although qualitative phytochemical screening revealed that both ethanol and DES extracts contained similar classes of secondary metabolites, including phenolics, flavonoids, terpenoids, and saponins, quantitative analyses demonstrated clear differences between the two extraction systems. The DES extract exhibited both a higher total phenolic content and stronger antioxidant activity than the ethanol extract. These findings suggest a positive relationship between total phenolic content and antioxidant activity. The higher phenolic concentration recovered by the DES was accompanied by enhanced DPPH radical-scavenging capacity, supporting the important role of phenolic compounds as the major contributors to the antioxidant activity of *C. equisetifolia* stem bark extracts.

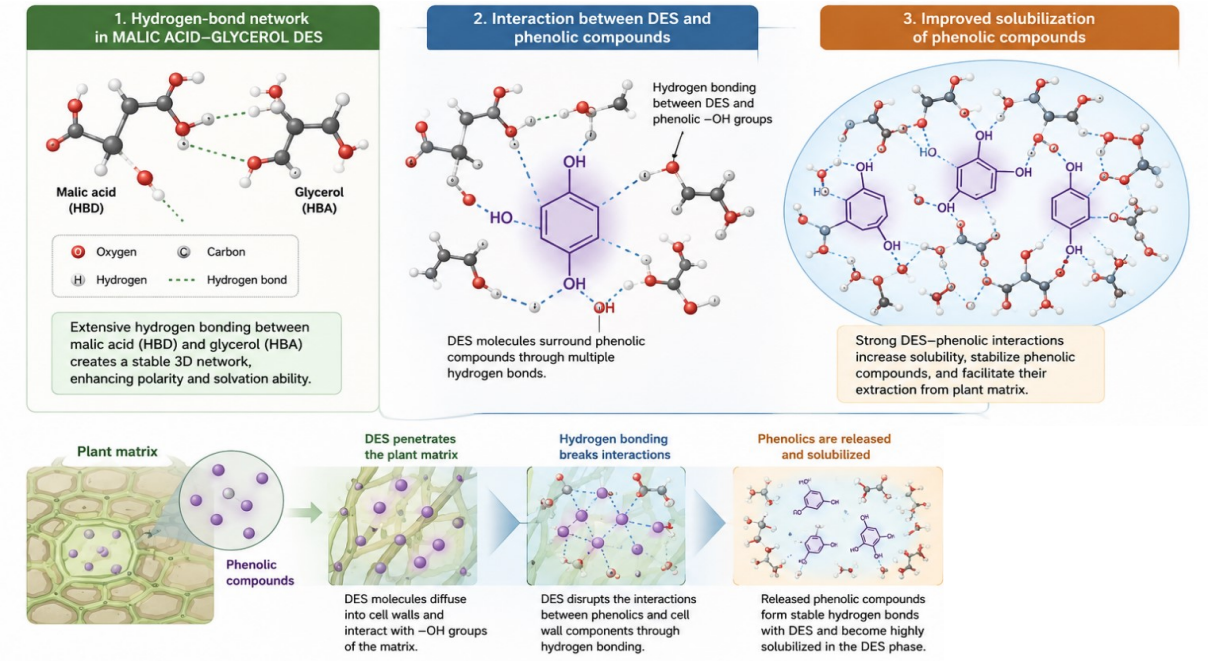


Figure 4. Schematic illustration of the proposed hydrogen-bonding interactions between the malic acid–glycerol deep eutectic solvent (DES) and phenolic compounds from Casuarina equisetifolia stem bark.

The superior extraction performance of the DES is likely associated with the hydrogen-bond interactions established between the solvent components and phenolic constituents in the plant matrix as shown in Figure 4. Such interactions improve the solubilization of hydroxyl-containing compounds and facilitate their release from plant tissues, resulting in greater recovery of antioxidant molecules than conventional ethanol extraction. Overall, these results demonstrate that the synthesized malic acid–glycerol DES not only successfully formed a stable eutectic system, as confirmed by FTIR analysis, but also enhanced the extraction of phenolic antioxidants from *C. equisetifolia* stem bark. Consequently, the DES extract exhibited stronger antioxidant activity than the ethanol extract, highlighting the potential of malic acid–glycerol DES as a sustainable green extraction solvent for obtaining antioxidant-rich botanical extracts intended for cosmetic applications.

CONCLUSIONS

This study demonstrated that the malic acid–glycerol deep eutectic solvent (DES) was successfully synthesized, as confirmed by FTIR analysis, and effectively extracted antioxidant compounds from *Casuarina equisetifolia* stem bark. Both ethanol and DES extracts contained phenolics, flavonoids, terpenoids, and saponins; however, the DES extract exhibited a higher total phenolic content (50.3 mg GAE g^{-1} extract) and stronger antioxidant activity (IC_{50} = 42.2 ppm) than the ethanol extract (42.2 mg GAE g^{-1} extract; IC_{50} = 54.6 ppm). These findings indicate that the hydrogen-bonding characteristics of the malic acid-glycerol DES enhanced the extraction of phenolic antioxidants, highlighting its potential as a green extraction solvent. Overall, *C. equisetifolia* stem bark represents a promising source of natural antioxidants for cosmetic applications, although further studies are required to identify the individual bioactive compounds and evaluate their biological efficacy.

Acknowledgements: This research is supported by Institut Teknologi Sumatera under Research Grant: Luaran Wajib Hibah Penelitian Itera Skema Penelitian Kepakaran Tahun 2026 No. Kontrak 2269bp/DST/IT9.2.1/PT.01.03/2026

Authors' Contributions: Herayati designed the study. Devika Damayanti Silalahi carried out the laboratory work. Herayati, Devika & Indah analyzed the data. Herayati wrote the manuscript. All authors read and approved the final version of the manuscript

Competing Interests: The authors declare that there are no competing interests.

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