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Identification of Bioactive Ingredients of Heath Forest's Sedges Species as Potential Natural Poison

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Abstract

Heath forests, also called *kerangas* forests, have a different composition and structure compared to the adjoining dipterocarp forests. They are found throughout the tropic regions and grow well in nutrient-poor sandy soil. Hence, there is a need to carry out a comprehensive analysis of the constituent composition of the plant species growing in these forests as it would help the researchers to determine the complete potential of every species. In this study, the researchers have attempted to analyze the functional groups of the compounds present in the various sedge plant species present in the heath forests, such as *Lepironia articulata*, *Dapsilanthus disjunctus* and *Eleocharis ochrostachys*. For this purpose, they used the FT-IR analysis technique for determining the functional groups at absorbance values ranging between 400 and 4000 cm-1. Then, they identified the phenolic compounds and individual phenolic acids with the help of a High-Performance Liquid Chromatography technique coupled with the photo Diode Array Detector (HPLC-DAD). In the past, the sedges were recognized for their valuable traditional properties as they contained several bioactive compounds. In this paper, the researchers extracted the water-soluble compounds from the sedge species using an alkaline technique, where they used sodium hydroxide (NaOH) for extraction of bioactive compounds for 12h in the oven at 60°C, followed by an acidic treatment using hydrochloric acid (HCl, pH 2). Thereafter, they re-extracted the supernatant using different types of solvents. The results of these experiments indicated that the sedge species contained 5 major functional groups, i.e., aliphatic ethers (1050-1150 cm^{-1}), alcohols (1200 cm^{-1}), aromatic hydrocarbon (1700-2000 cm^{-1}), aliphatic ketones (1705-1725 cm^{-1}) and aliphatic hydrocarbons (2950-2975 cm^{-1}). The researchers characterized the phenolic compounds present in the sedge species based on their retention time and then compared the UV data with the different standards that were used. It was noted that *Lepironia articulata* contained the maximal Total Phenolic Content (984.63 ± 5.96 µg GAE/g DW) out of all the sedge species that were studied. Also, trans-p-Coumaric acid was seen to be the main phenolic compound in *Eleocharis ochrostachys* (645.75 µg/g dw) whereas Vanillic acid was the main phenolic compound present in *Dapsilanthus disjunctus* (138.72 µg/g dw) and *Lepironia articulata* (114.72 µg/g dw). The results indicated that Caffeic acid, Ferulic acid, 4-Hydroxybenzoic acid, Vanillic acid and trans-p-Coumaric acid were present in high concentrations in the sedge species. Out of these compounds, trans-p-Coumaric was the main phenolic compound that was present in the sedge plant extracts. In this study, the researchers have described the uses of the FT-IR and HPLC analyses and also attempted to garner the interest of the researchers in the field of natural plant analysis.

Keywords: Allelopathy, *Dapsilanthus disjunctus*, *Eleocharis ochrostachys*, heath forests, *Lepironia articulata*, natural poison

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1. Introduction

Heath forests are known as *kerangas* forests or *kerapah* forests. The plant species belonging to these forests have a completely different composition and structure compared to the adjoining dipterocarp forests [1]. The *kerangas* forests found in the Malaysian region of Borneo have been extensively studied in the past; while the plant species in the Indonesian *kerangas* forests were only studied for their flora by the researchers who were conducting soil and botanical explorations [1]. The heath forests are found throughout the tropical region as they thrive well in the nutrient-poor sandy soil (podzols). The plant species in these forests have a characteristic vegetation physiognomy, where they have a stunted appearance, short and non-tapered stems and sclerophyllous leaves [2]. The most popular plant species

found in the Kabili-Sepilok Forest Reserve, Sabah, were seen to be *Cotylelobium melanoxylon*, *Cleistanthus gracilis*, *Diospyros fusiformis*, *Dracaena elliptica*, *Gaertnera junghuhniana*, *Garcinia bancana*, *Pimelodendron griffithianum*, etc. [2]. It was noted that the farmers who used the conventional shifting cultivation techniques usually avoided burning the sedge plant species in the heath forests before their cultivation. Despite these measures, they were able to acquire sufficient quantities of food and medicinal plants for consumption [3]. One such region, i.e., Rantau Abang, located in Marang, Terengganu, Malaysia, is surrounded by the heath forest ecosystem and contained some traces of these forests. Though the *Melaleuca cajuputi* species were predominantly found in the heath forest [4], many other plant species like sedges existed in this forest ecosystem and are yet to be discovered and analysed. The different sedge species that have been found in the hedge forests are *Dapsilanthus disjunctus*, *Lepironia articulata* and *Eleocharis ochrostachys*. Out of these species, *L. articulata* (called rumput kercut in Malay) belonged to the Cyperaceae family and was found abundantly in the heath forest, Terengganu as well as Tasik Bera, Pahang [5]. It is a pioneer producer that grows in the tropical swamp area in West Malaysia [6]. Furthermore, *D. disjunctus* (or *Leptocarpus disjunctus*, also called rumput kuda in Malay) [7] belongs to the Restionaceae family. It is an edible plant and is generally consumed as a vegetable even though it causes dizziness as a side effect [8]. *Eleocharis ochrostachys* (called rumput sikat in Malay) belonged to the Cyperaceae family [9].

Many plant species contain the phenol compound, wherein the Hydrogen group present in the compound is replaced by the hydroxyl group. The phenolic compounds are regarded as a type of prototypical poison that enters the body after inhalation through the mucous membranes and skin. Thereafter, it leads to the production of many insoluble proteins, further affecting different cellular activities, especially in the nervous system. [10]. The natural phenolic compounds are often seen to be an end product in the acetate and shikimate pathways. They range between simple molecules (like phenolic acids, flavonoids, phenylpropanoids) and highly-polymerized compounds (melanin, lignin, tannins) [11]. The differences noted between the quantitative and qualitative composition of the phenolic compounds could be attributed to the genetic differences amongst the plant species, growth conditions, environments and plant maturation stages [12]. Though many researchers have investigated the bioactivities of the phenolic acid compounds, very few have analyzed the functions and bioactivities of the metabolite compounds, particularly since a majority of the compounds are not available commercially [13].

Some plant species also produce phenolic compounds, like phenolic acids for inhibiting the growth of the competitive plant species (allelopathy) [14]. Allelopathy plays both a stimulatory and inhibitory role in different plant processes like plant growth and development, disease/weed management, seed germination, cell division, reproduction, or biosynthesis of photosynthetic pigments of other plant species, where the plants release some allelochemicals, in the form of secondary metabolites [15]. Phenolic acids, like gallic acid, protocatechuic acid (3,4-dihydroxybenzoic acid), chlorogenic acid, 3,5-dinitrobenzoic acid, p-hydroxybenzoic acid, 3,4-dihydroxybenzaldehyde and caffeic acid (3,4-dihydroxycinnamic acid) that are present in the aqueous plant extracts, show a higher allelopathy effect [16].

In this study, the researchers analysed the functional groups of the compounds extracted from the heath forest sedge species, such as *Lepironia articulata*, *Dapsilanthus disjunctus* and *Eleocharis ochrostachys*. For this purpose, they carried out an FT-IR analysis, where the samples were analysed at different absorbance values ranging between 400-4000 cm-1. Thereafter, they detected the various phenolic acids in the extracts with the help of a High-Performance Liquid Chromatography coupled with the photodiode Array Detector (HPLC-DAD).

2. Materials and methods

2.1. Plant Materials and Sample Preparation

In this study, the researchers collected the different plant sedge species, such as *Lepironia articulata* (rumput kercut), *Dapsilanthus disjunctus* (rumput kuda) and *Eleocharis ochrostachys* (rumput sikat) from heath forest of Terengganu. Thereafter, the sedge species were authenticated at the Herbarium Unit, Department of Landscape Architecture, Kulliyyah of Architecture and Environmental Design, International Islamic University, Malaysia. For identifying the phenolic acids present in the sedge extracts using the HPLC analysis, the researchers purchased the phenolic acid standard compounds (Caffeic acid, 2-Coumaric acid, 4-Coumaric acid, Ferulic acid, Hydroxybenzoic acid, trans-p Coumaric acid and Vanillic acid) from Sigma Aldrich (USA).

2.2. Sequential Alkaline Extraction of Sedges Species

The researchers extracted the phenolic compounds present in the sedge species using a sequential alkaline extraction technique described earlier [17] with some modifications. Sequential alkaline extraction refers to a technique that can be used for extracting the free and bound phenolic compounds from the plant samples using sodium hydroxide (NaOH) for 12h at 60°C in an oven. Then, these alkaline extracts were treated using hydrochloric acid (HCl) till the pH was 2. The samples were then centrifuged and the supernatant was extracted and re-extracted with different solvents like hexane, ethyl acetate, butanol and ethanol. The

solvent extracts were dried and the dried samples were resuspended in methanol for HPLC analysis.

2.3. Identification of Phenolic Functional Groups

The researchers determined the functional groups present on the methanolic extracts using the FT-IR spectrometer (Nicolet iS50 FT-IR Spectrometer; Thermo Scientific, USA), where the samples (500 µl poured on the ATR crystal [18] were analyzed at the absorbance values ranging between 400-4000 cm^{-1}. The researchers used the OMNIC ver. 9.2 (Thermo Scientific) software for analyzing the FT-IR data.

2.4. Determination of Total Phenolic Content (TPC)

The researchers determined the TPC of the above extracts using the Folin-Ciocalteu technique as mentioned earlier [19] with a few modifications. The TPC was quantified using the hydrolyzed extracts. The TPC values were expressed in the form of Gallic Acid Equivalence (GAE) per gram dry weight of the sample, after determining the values using the TECAN microplate reader.

2.5. Identification of Individual Phenolic Compounds

As for determining the individual phenolic acid compounds present in the extracts, the researchers analyzed the samples using the HPLC instrument (Agilent Technologies, Palo Alto, CA, USA), comprising of a binary pump, auto sampler injector, and a Diode Array Detector (DAD). The extracts were analyzed using the Zorbax SB-C18 (Eclipse 100 × 2.1 mm, 1.8 µm) Reverse Phase column (Agilent Technologies, USA) that was operated at 25°C throughout the HPLC run. For analyzing the extracts, the researchers used a linear gradient elution technique having 2 mobile phases, where Phase A consisted of 1% formic acid dissolved in water/ acetonitrile (90:10 v/v); while Phase B was Acetonitrile (100%). The following elution gradient was used for analysis: a linear gradient for 0-20 mins with 0% B to 40% B; linear gradient for 20-25 mins with 40% B to 60% B; linear gradient with 25 to 25.1 mins with 60-100% B; linear gradient from 25.10-35 mins with 100% B; and a linear gradient from 35-35.1 mins with 100% B to 0% B solvent. The samples were eluted at the flow rate of 0.4 ml/min while the phenolic acids were detected at 280 nm throughout the elution gradient [20]. The researchers identified the phenolic acids based on their retention times and UV spectra, compared to the standard compounds.

2.6. Statistical analysis

The researchers statistically analyzed the results determined in this study. The concentrations of the phenolic acid compounds determined in this study were represented as the Mean ± standard deviation since all experiments were conducted in triplicates. The researchers determined the One-way Variance analysis (ANOVA) and Tukey's test using the XLSTAT-Pro (2014) software (Addinsoft, France). All the tests indicated that mean concentrations of phenolic acids were significant at the 99% confidence level ($p<0.001$).

3. Results and discussion

3.1. Analysis of Phenolic Functional Groups

The FT-IR spectroscopic analysis provides a lot of relevant information regarding the probable functional groups present on the active compounds. In this study, the researchers analyzed the functional groups of the active compounds present in the dried leaf extracts of the 3 sedge species, i.e., *Lepironia articulata*, *Dapsilanthus disjunctus* and *Eleocharis ochrostachys*. The plant species were subjected to a sequential alkaline extraction with fractional distillation, followed by the FT-IR analysis. Table 1 presents the results of functional group analysis. In the table, the researchers also presented the comparison of the absorption peak values with the values included in the FT-IR spectroscopy library. The results indicated that the plant species contained 5 major chemical groups, i.e., aliphatic ethers (1050-1150 cm^{-1}), alcohols (1200 cm^{-1}), aromatic hydrocarbon (1700-2000 cm^{-1}), aliphatic ketones (1705-1725 cm^{-1}) and aliphatic hydrocarbons (2950-2975 cm^{-1}). Furthermore, the FT-IR analysis of all different solvent extracts acquired after sequential alkaline extraction of the dried *D. disjunctus* leaves indicated the presence of various functional groups (Table 1). Firstly, the hexane fraction contained the main peak at 1711.28 cm^{-1} and 2956.28 cm^{-1}. The characteristic peak noted at 2950-2975 cm^{-1} was assigned to the C=O stretch (aliphatic ketones); while the peak noted at 1700-1725 cm^{-1} was assigned to the CH_3 asymmetric bond (aliphatic hydrocarbons). Secondly, the ethyl acetate fraction also showed the main peak at 1603.55 cm^{-1}. The characteristic peak noted at 1700-2000 cm^{-1} could be assigned to the C=C vibration (aliphatic hydrocarbons). Finally, the ethanolic fractional extract indicated the presence of 2 main peaks at 1051.67 cm^{-1} and 1219.42 cm^{-1}. The main peak noted at 1050-1150 cm^{-1} was assigned to the C-O stretch (aliphatic hydrocarbon); whereas that at 1200 cm^{-1} was assigned to the -C-O stretch and -OH deformation (alcohols). However, the butanol extract did not show the presence of any functional group. Thus, 3 functional groups were noted after the FT-IR analysis in the *D. disjunctus* extracts.

When the researchers carried out the FT-IR analysis of the sequential alkaline extracts derived from the dried *L. articulata* leaves, the hexane extracts showed the main peak at 2956.54 cm^{-1}. The peak noted at 2950-2975 cm^{-1} was assigned to the CH_3 asymmetric bond (aliphatic hydrocarbon). The ethanolic extract displayed 2 major peaks at 1125.15 cm^{-1} and 1221.01 cm^{-1}. The peak noted at 1050-

1150 cm^{-1} was assigned to the C-O stretch (aliphatic ether) while that noted at 1200 cm^{-1} was assigned to the -C-O stretch and -OH deformation (alcohol). The ethyl acetate and butanol fractions did not contain any functional group. Thus, the *L. articulata* extracts showed the presence of 3 functional groups.

Next, the FT-IR analysis of the sequential alkaline extracts derived from the dried *E. ochrostachys* leaves, the hexane fraction showed the presence of the main peak at 2956.78 cm^{-1}. The peak that was detected at 2950-2975 cm^{-1} was assigned to the CH_3 asymmetric (aliphatic hydrocarbon). The butanol fractional extract displayed the main peak at 2963.99 cm^{-1}. The peak at 2950-2975 cm^{-1} was assigned to the CH_3 asymmetric bond (aliphatic hydrocarbons). The ethanolic fraction contained the main peak at 1092.74 cm^{-1}. The characteristic peak noted at 1100 cm^{-1} was assigned to the C-O stretch and –OH deformation (alcohols). However, the ethyl acetate fraction did not contain any functional group. Thus, 2 functional groups could be detected after the FT-IR analyses of the *E. ochrostachys* leaf extracts.

In conclusion, the sedge species showed the presence of many functional groups. The *D. disjunctus* dried leaf solvent fractions, except butanol, showed the presence of different functional groups, like aliphatic hydrocarbons, aliphatic ketones, aromatic hydrocarbons and alcohol. However, the ethyl acetate fraction of the *E. ochrostachys* leaves did not contain any functional group; while the ethyl acetate and butanol fractions of *L. articulata* leaves did not contain any functional groups.

3.2. Analysis of Phenolic Compounds and Individual Phenolic Acids in The Sedge Species

The Folin Ciocalteu assay was used for assessing the Total Phenolic Content (TPC) present in the 3 sedge species collected from the heath forest, which was then expressed as gallic acid equivalent (i.e., µg GAE/g DW). The results indicated that the *L. articulata* contained the maximal TPC value (984.63 ± 5.96 µg GAE/g DW), followed by the *D. disjunctus* (784.97 ± 7.03 µg GAE/g DW) and *E. ochrostachys* species (22.88 ± 0.14 µg GAE/g DW) (Table 2). Figs. 1-3 present the HPLC analysis results of the individual phenolic compounds. It was noted that the sequential alkaline extracts of the *E. ochrostachys* leaves contained the highest concentration of trans-p-Coumaric acid (645.75 µg/g DW) (Fig. 1), whereas the *D. disjunctus* and *L. articulata* leaf extracts contained the maximal concentration of vanillic acid, i.e., 138.72 µg/g DW (Fig. 2) and 114.72 µg/g DW (Fig. 3), respectively. Interestingly, it was seen that the 5 major phenolic acids, i.e., Caffeic acid, 4-Hydroxybenzoic acid, Ferulic acid, Vanillic acid and trans-p-Coumaric acid were found abundantly in the sedge species.

These results indicated that the *E. ochrostachys* species contained a higher number of phenolic acids compared to the *D. disjunctus* and *L. articulata* species. It was seen that the trans-p-Coumaric acid concentration was higher in the *E. ochrostachys* extracts, in addition to other phenolic acids like Ferulic acid (642.25 µg/g DW), Caffeic acid (117.25 µg/g DW), Vanillic acid (108.50 µg/g DW), 2-Coumaric acid (52.85 µg/g DW), 3-Coumaric acid (31.33 µg/g DW) and 4-Hydroxybenzoic acid (5.85 µg/g DW) (Fig. 1). As presented in the results, the ethanolic extracts contained the maximal concentration of phenolic acids, followed by the ethyl acetate, hexane and butanol extracts of *E. ochrostachys* leaves. It was also seen that out of the different phenolic acid compounds, the concentration of Vanillic acid was higher in the *D. disjunctus* extracts, followed by the trans-p-Coumaric acid (86.16 µg/g DW), Ferulic acid (36.93 µg/g DW), Caffeic acid (26.25 µg/g DW) and 4-Hydroxybenzoic acid (25.32 µg/g DW) concentrations (Fig. 2). The results indicated that the ethyl acetate extract contained a higher phenolic acid concentration, followed by the ethanol, hexane and butanol extracts. The *D. disjunctus* extracts did not show the presence of the 3-Coumaric acid and 2-Coumaric acids. Furthermore, it was noted that the *L. articulata* extracts contained a higher Vanillic acid content compared to other phenolic acids, followed by the trans-p-Coumaric acid (34.44 µg/g DW), 4-Hydroxybenzoic acid (23.45 µg/g DW), Caffeic acid (18.03 µg/g DW) and Ferulic acid (2.78 µg/g DW) concentrations. The ethyl acetate fraction of the *L. articulata* species showed the highest concentration, followed by the ethanol, hexane and butanol extracts.

In conclusion, the results indicated that the trans-p-Coumaric and Vanillic acids were the main phenolic compounds present in the sedge species extracts. However, 3-Coumaric acid and 2-Coumaric acids could not be detected in the *D. disjunctus* and *L. articulata* extracts. Ethyl acetate and ethanol were seen to be the best solvents that could be used for extracting the phenolic acids from the sedge species. It was concluded that owing to the extensive distribution of phenolic compounds and the presence of phenolic compounds in the sedge species, the natural polyphenols could be used owing to their neuroprotective activities, antimutagenic, anti-inflammatory, antioxidant activity, and/or anticarcinogenic activities [21]. HPLC is widely used for separation technology as it helps in detecting and quantifying the different phytochemical compounds in the plant extracts [22]. Furthermore, the presence of phenolic acids in the plant extracts highlights their inhibitory activity against the gram-negative and gram-positive pathogenic microorganisms [23]. The phenolic acids can be used for decreasing the consumption of different synthetic fungicides, thereby decreasing their environmental effect [24].

Table 1: FT-IR peak values of *Dapsilanthus disjunctus, Lepironia articulata* and *Eleocharis ochrostachys* in sequential alkaline extraction from hexane, ethyl acetate, butanol and ethanol

Sedge species	Solvent	Peak (wave number cm^{-1})	Intensity	Bond	Functional group	Group frequency (cm^{-1})
Dapsilanthus disjunctus	Hexane	1711.28	99.031	C=O stretch	Aliphatic ketones	1705 - 1725
		2956.28	98.348	CH_3 asymmetric	Aliphatic hydrocarbon	2950 - 2975
	Ethyl acetate	1603.55	98.744	C=C vibration	Aromatic hydrocarbon	1700 - 2000
	Ethanol	1051.67	91.900	C-O stretch	Aliphatic hydrocarbon	1050 - 1150
Lepironia articulata	Hexane	2956.54	99.432	CH_3 asymmetric	Aliphatic hydrocarbon	2950 - 2975
	Ethanol	1125.15	97.618	C-O stretch	Aliphatic ether	1050 -1150
		1221.01	97.947	-C-O stretch & -OH deformation	Alcohol	1200
Eleocharis ochrostachys	Hexane	2956.78	99.728	CH_3 asymmetric	Aliphatic hydrocarbon	2950 - 2975
	Butanol	2963.99	99.593	CH_3 asymmetric	Aliphatic hydrocarbon	2950 - 2975
	Ethanol	1092.74	57.526	-C-O stretch & -OH deformation	Alcohol	1100

Table 2: Total phenolic content (µg GAE/g DW) for *D. disjunctus, L. articulata* and *E. ochrostachys*

Sedge species	**Total phenolic content (µg GAE/g DW)**
Dapsilanthus disjunctus	784.97 ± 7.03
Lepironia articulata	984.63 ± 5.96
Eleocharis ochrostachys	22.88 ± 0.14

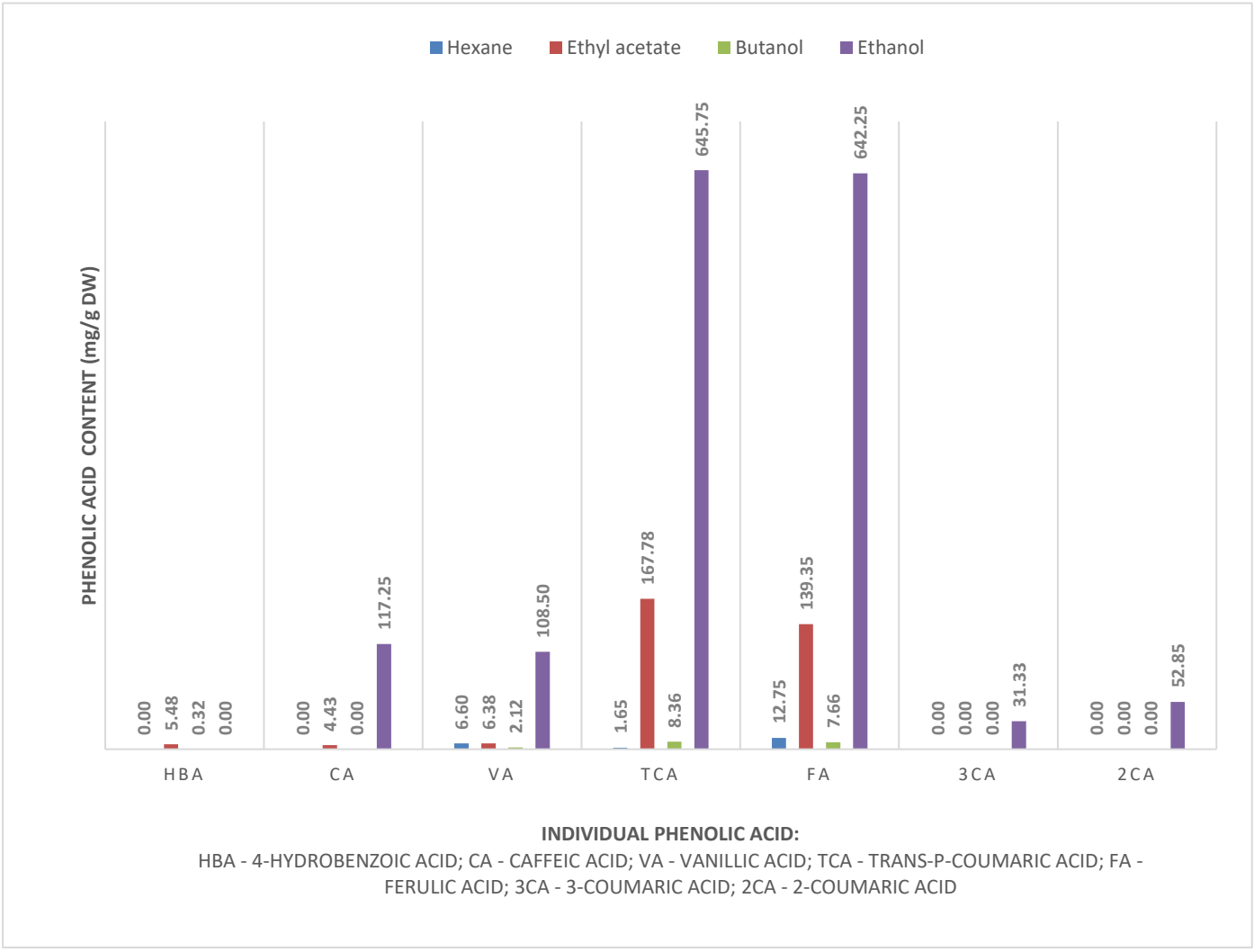

Fig. 1. Phenolic acid content of *E. ochrostachys* in sequential alkaline extraction from hexane, ethyl acetate, butanol and ethanol

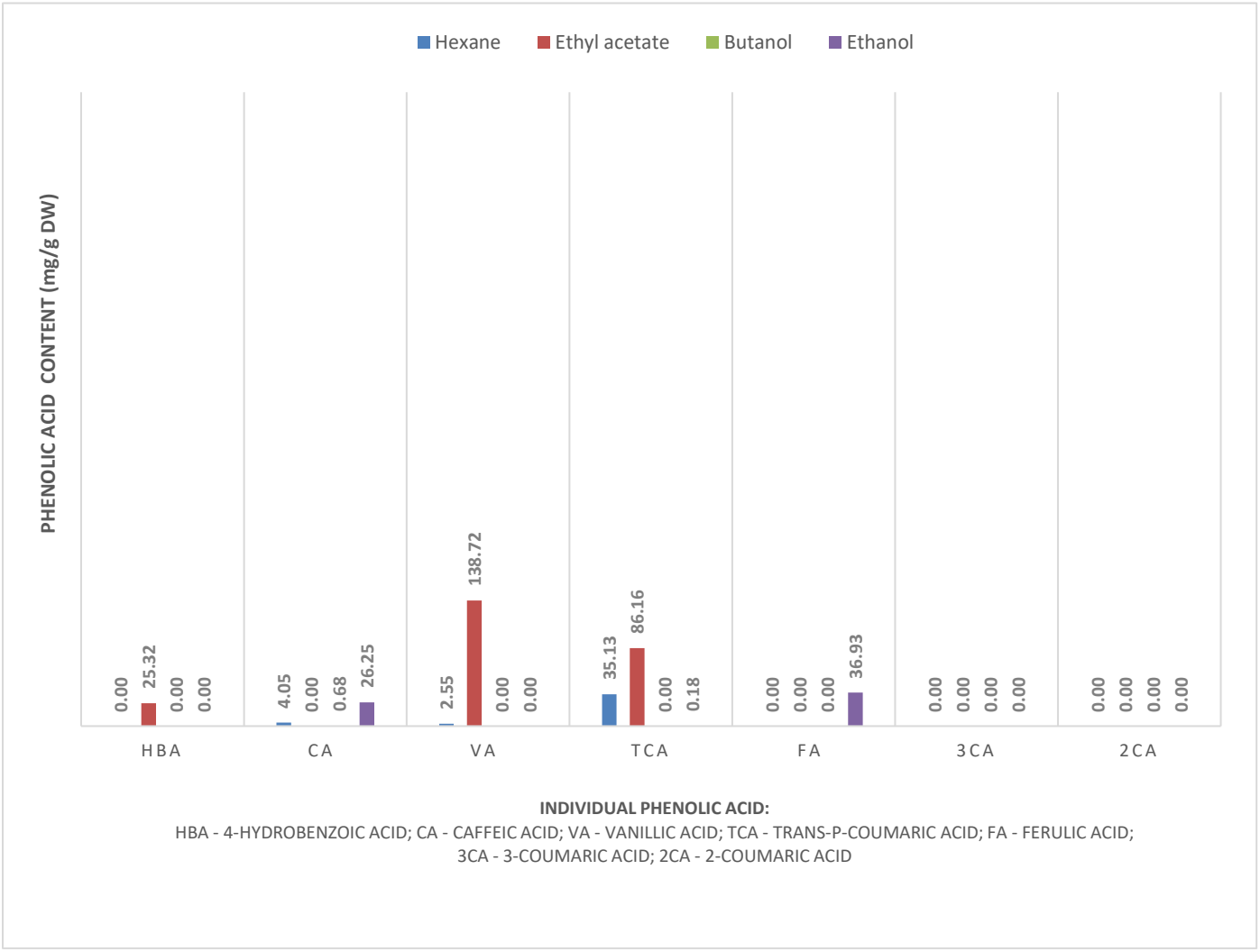

Fig. 2. Phenolic acid content of *D. disjunctus* in sequential alkaline extraction from hexane, ethyl acetate, butanol and ethanol

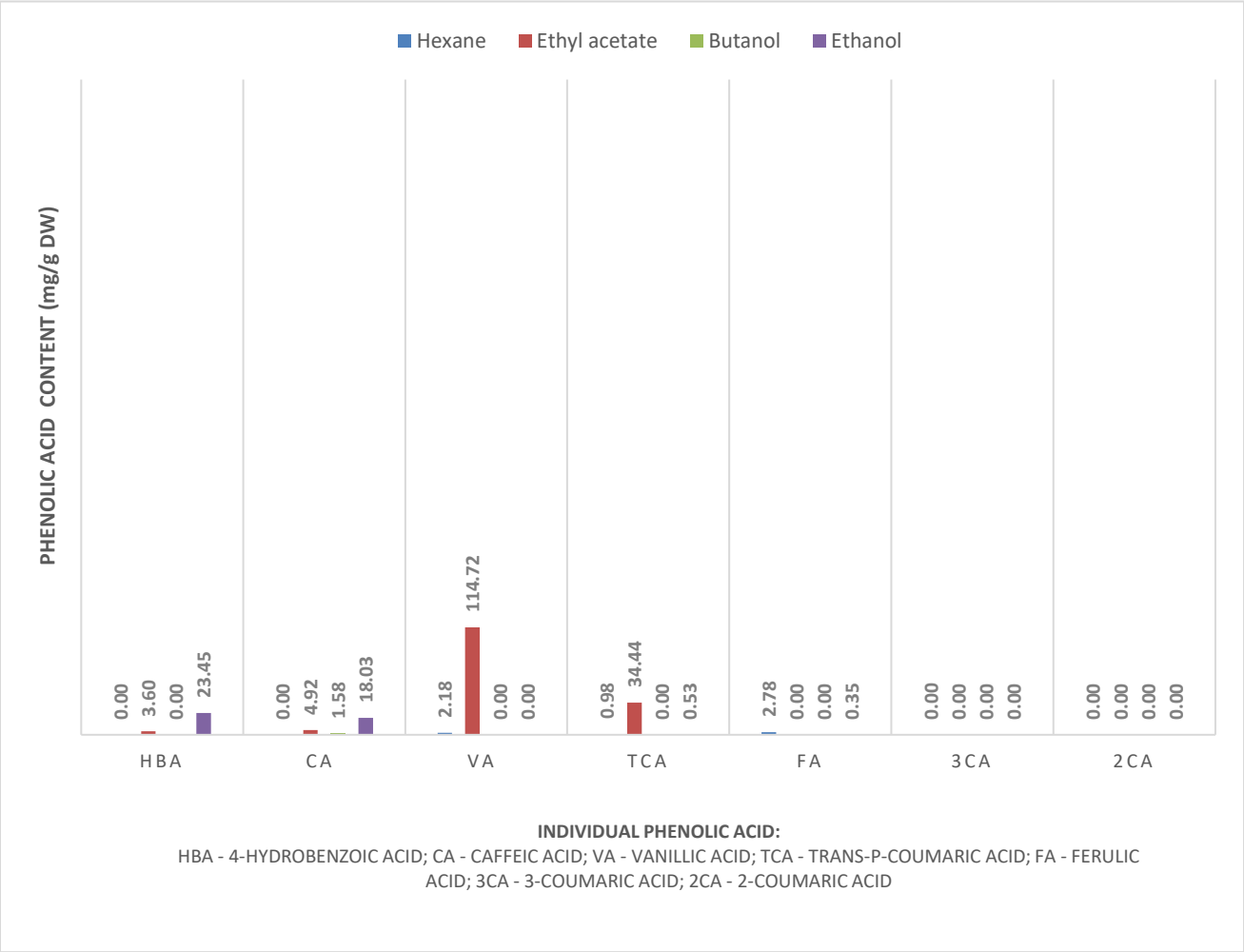

Fig. 3. Phenolic acid content of *D. disjunctus* in sequential alkaline extraction from hexane, ethyl acetate, butanol and ethanol

Additionally, the phenolic compounds affect the membrane potential, which affects the exchange of external and internal components in the cells [25]. Thus, the phenolic acids have a higher biological significance and need to be studied for determining their role in preventing the onset of diseases [14]. Phenolic acids, like Ferulic acid, have shown high antibacterial activity against many pathogenic microbes. The antibacterial activity of a compound is based on its Minimum Inhibitory Concentration (MIC) [26]. In the past, the researchers also noted that p-Hydroxybenzoic acid showed higher antioxidant activity against the free radicals, along with high antimicrobial activity against the bacterial and fungal species. p-Hydroxybenzoic acid also displayed a higher estrogenic and antimutagenic activity [27]. In [28], the researchers tested the antibacterial properties of the phenolic acids like cinnamic, p-hydroxybenzoic and p-coumaric acids and compared these activities against those displayed by their glucuronate protected forms and methylated metabolites. In [29], the researchers noted the presence of phenolic compounds like p-coumaric, gentisic, gallic, p-hydroxybenzoic, syringic, vanillic acids and catechol in *Eucalyptus tereticornis, E. camaldulensis, E. polycarpa* and *E. microtheca* species that could be used as allelopathic chemical compounds. In a different study, the researchers noted that the ethanolic extract of the *D. disjunctus* species showed a similar efficiency to the known antianxiety drugs and affected locomotor activity [8].

4. Conclusions

In this study, the researchers highlighted the fact that the FT-IR and HPLC analyses could be used for detecting the presence of many phytochemical compounds in different extracts. Here, the researchers carried out a preliminary analysis where they detected the presence of 5 functional groups in the selected 3 sedge species. However, the sedge species need to be investigated further for determining the presence of additional bioactive compounds that could be

used for different applications. Many chromatography and purification techniques need to be implemented for isolating the bioactive compounds in future. As indicated by the results of this study, the sedge species can be used as an additional or alternative source of phenolic compounds and also potential as natural herbicide or poison.

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