Original Research Article

Antioxidant and Toxicity Assay-guided Isolation of Herniarin from *Equisetum debile* Roxb. (Equisetaceae)

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ABSTRACT

Introduction: Bioassay-guided isolation of the bioactive components from *Equisetum debile* Roxb. ("sumbak") was conducted. **Objectives:** This study aimed to isolate and characterize bioactive components from *E. debile*. **Materials and Methods:** Ethanolic extract of whole plant sample of *E. debile* was sequentially partitioned to obtain the bioactive choroform-soluble (EdC) partition. EdC was purified *via* chromatographic techniques. Fractions obtained after purification were analyzed for cytotoxic and antioxidant properties. Brine shrimp lethality as well as *in vitro* antioxidant assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging, Total Antioxidant Capacity and Total Phenolics Content assays were carried out. One-dimensional (1D) and two-dimensional NMR and LC-MS analyses were conducted to elucidate structure of the isolated compound. **Results and Discussion:** The whole plant sample of *E. debile* afforded herniarin. **Conclusion:** The present findings imply that *E. debile* can be a potential source of bioactive components.
Key words: *Equisetum debile*, Herniarin, 7-methoxycoumarin, Bioassay-guided isolation.

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INTRODUCTION

Equisetum debile Roxb., commonly known as sumbak is found in the Philippines, particularly, in Bukidnon, Davao provinces and in Benguet. *E. debile* Roxb. has been administered as a cooling medicine, given for the treatment of gonorrhea and bone fractures. The decoction of the plant has been used for nasal polypus, cancer of breast, liver, intestine, stomach, kidneys and tongue.[1] Moreover, ethnomedical reports in Nepal have shown that the plant stem juice is given for gonorrhea and plant root juice is given for urinary troubles, sprains, fractures, burns and scabies.[2] Investigations and surveys have also cited that whole plant has been used to cure jaundice and hepatitis.[3] In Indonesia, boiled leaves of *E. debile* has been used to overcome kidney stones.[4] Recently, cytotoxicity and antimicrobial studies revealed that the n-hexane-, ethyl acetate- and n-butanol extract of *E. debile* aerial stems exhibited quite potent activity in brine shrimp lethality bioassay.[1] Moreover, results of a preliminary study indicated that the chloroform-soluble extract of *E. debile* exhibited potential cytotoxicity against the brine shrimp nauplii and antioxidative properties in terms of DPPH radical scavenging, total antioxidant capacity and total phenolics content.[5] These results suggests that *E. debile* might contain cytotoxic and antioxidant chemical compounds.

This study reports on the isolation and structure elucidation of herniarin from the whole plant sample of *E. debile*. A number researches have reported anti-dermatophytic and antimicrobial properties of herniarin.[6,7] Recently, its effect on human bladder cancer cells was investigated. Results have shown that herniarin, a nontoxic agent, had enhancing effects on cisplatin cytotoxicity on TCC (Transitional Cell Carcinoma) in

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different concentrations, *in vitro* and *in vivo*.[8] Herniarin was found to reduce viability and migration of RK33 LCC (human laryngeal cancer cells) in a dose-dependent manner. It has also induced apoptosis of LCC which suggests its therapeutic potential in the treatment of laryngeal cancer.[9]

Although herniarin has been isolated from plant species such as flowers of *Matricaria chamomilla* L., leaves of pineapple weed (*Matricaria matricarioides*), some members of the Asteraceae family and different *Lavandula* species,[10] to the best of our knowledge this is the first report on its isolation from *E. debile*.

MATERIALS AND METHODS

General Experimental Procedure

NMR and LC-MS experiments were performed at the University of Florida, Gainesville, Florida, United States of America. The one-dimensional (1D) proton NMR (^{1}H-NMR) and the two-dimensional (2D) ^{1}H-^{1}H Homonuclear Correlation (COSY), ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC) and ^{1}H-^{13}C Heteronuclear Multi-Band Correlation (HMBC) spectra were recorded using Avance II 600 (600 MHz) while ^{13}C NMR spectra were recorded using Avance III 600 (150 MHz). NMR spectra were obtained in CD_3OD. Chemical shifts are reported in ppm (δ) and referenced to residual CD_3OD and D_2O (δ_H = 3.34 ppm and δ_H = 4.91 ppm). Measurement of the molecular weight of the isolate was done *via* LC-MS (Agilent Series 1100 (G1314A). Absorbance measurements were carried out on a double beam UV-Vis spectrophotometer (Lasany-LI-2800). Gravity column chromatography was employed using silica gel 60 (70-230 mesh, Scharlau). TLC was performed with plastic-backed plates coated with silica gel F_{254} (Merck KGaA, Darmstadt, Germany) and the plates were visualized by viewing under the UV light at 254 nm and 365 nm, exposure to iodine crystals and/or spraying with vanillin/sulfuric acid reagent, followed by warming.

Plant Material

Whole plant **s**amples of *Equisetum debile* Roxb. were collected from Kalasungay, Malaybalay, Bukidnon and authenticated by a taxonomist from the Biological Sciences Department, MSU-IIT. The plant samples were then washed with tap water, rinsed with distilled water and air-dried.

Extraction and Isolation

About 2 kg of air-dried plant samples was pulverized, weighed and soaked with 95% ethanol for 3 days. To obtain the crude ethanol extract (EdE), the resulting solution was then filtered, concentrated *in vacuo* at temperature not exceeding 40°C and weighed. A portion of the EdE (48.1686 g) was then sequentially partitioned in hexane: water (1:1) and chloroform: water (1:1) solutions. The hexane-soluble, chloroform-soluble and water-soluble portions were individually concentrated *in vacuo* and weighed to give hexane - (EdH), chloroform -(EdC) and aqueous-soluble (EdA) extracts, respectively. The EdC (1.7988 g), which can be potential source of natural cytotoxic and antioxidant compound,[5] was subjected to silica gel chromatography using increasing proportions of chloroform in hexane (20% increment) and methanol in $CHCl_3$ (10% increment) to afford 12 fractions (EdC1-12). The fraction eluted with 10% MeOH in $CHCl_3$ (611.5 mg), which gave the best results for BSLA and *in vitro* antioxidant assays, was rechromatographed with $CHCl_3$ in hexane (20% increment), EtOAc in $CHCl_3$ (10% increment) and MeOH in EtOAc (20 % increment) to afford 9 sub-fractions. The sub-fraction eluted with 20% EtOAc in $CHCl_3$ (122.9 mg) was washed with chloroform and recrystallized with MeOH to yield 38.1 mg of needle-like colorless crystals of herniarin (Figure 2).

Brine Shrimp Lethality Assay (BSLA)

The assay was carried out employing the principle and protocol previously described by McLaughlin and Krishnaraju with slight modifications in the dose of extract tested.[11,12] Due to limitations in the weight of fractions obtained after chromatography, toxicity of fractions was tested at a single dose of 500 mg/L. A 0.50 mL of the 5000 mg/L fraction solution was placed in the test tube. Solvent was allowed to completely evaporate before the addition of the brine shrimp nauplii. Ten previously hatched brine shrimps were transferred to each test tube and sterilized sea water was added up to the mark corresponding to 5.00 mL. Control experiments using podophyllotoxin (positive) and seawater (negative) were simultaneously performed. The number of dead and alive nauplii was counted after 6 and 24 hrs for acute and chronic toxicity data, respectively. The results were evaluated and the percent mortality of the nauplii in each fraction was calculated.

DPPH Free Radical Scavenging Assay

The DPPH free radical scavenging activity of the *E. debile* extracts were determined by comparison with known antioxidant butylated hydroxytoluene (BHT) and ascorbic acid (AA).[13] A 3.00 mL of DPPH (0.1 mM) methanolic solution was added in to each of the 0.30 mL

of 500 mg/L sample solution. The mixtures were then shaken vigorously and allowed to stand under dark condition at room temperature for 1 hr. Using a UV-Vis spectrophotometer (Lasany-LI-2800), absorbance of the solutions was measured at 517 nm against methanol as a blank. Each determination was carried out in triplicate. The percent (%) DPPH inhibition was calculated according to the formula:

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

where:

$A_{control}$ = absorbance of the blank (methanol)

A_{sample} = absorbance of the extract or standard solution

Total Antioxidant Capacity (TAC) Assay

The total antioxidant activity of the sample was evaluated by the Phosphomolybdenum Method.[14] A 0.30 mL of 200 mg/L ethanolic sample solution was added with 3.00 mL of the reagent solution (0.6 M H2SO4, 28 mM sodium phosphate, 4 mM ammonium molybdate). The mixtures were then incubated at 95°C for 90 mins, allowed to cool to room temperature. Absorbance was measured at 695 nm using a UV spectrophotometer (Lasany-LI-2800). The total antioxidant activity of the various extracts was expressed as milligram Ascorbic Acid Equivalents per g sample (mg AAE/g sample) and milligram Butylated Hydroxytoluene Equivalents per gram sample (mg BHTE/g sample), which were derived from the calibration curve established using ascorbic acid and butylated hydroxytoluene as reference standards. All analyses were conducted in triplicates and results were reported as means of the triplicate analysis.

Total Phenolics Content (TPC) Assay

The total phenolics content of the sample solutions were determined by Folin-ciocalteu Method as described by Makkar *et al.*[15] In separate test tubes, 0.10 mL of 500 mg/L (in ethanol) sample solution was combined with 2.80 mL of 10% Na_2CO_3 and 0.10 mL of 2N Folin-Ciocalteu reagent. The mixture was then set aside for 40 mins before the absorbance was measured at 725 nm. The total phenolics content was measured as milligram Gallic Acid Equivalents per gram sample (mg GAE/g sample) which was derived from the linear equation of the calibration curve using various concentrations (25-, 50-, 100-, 200 mg/L) of gallic acid as working standards. Analysis was done in triplicates.

Table 1: Percent mortality of brine shrimp nauplii after 6 and 24-hr exposure to EdC fractions.

Fraction	% Mortality	
	After 6 hours	After 24 hours
EdC1	0.00	93.33
EdC2	3.33	100.00
EdC3	0.00	96.67
EdC4	0.00	30.43
EdC5	0.00	50.00
EdC6	0.00	10.00
EdC7	0.00	10.00
EdC8-12	n.d.*	n.d.*

* - not determined due to insufficient weight of fractions obtained

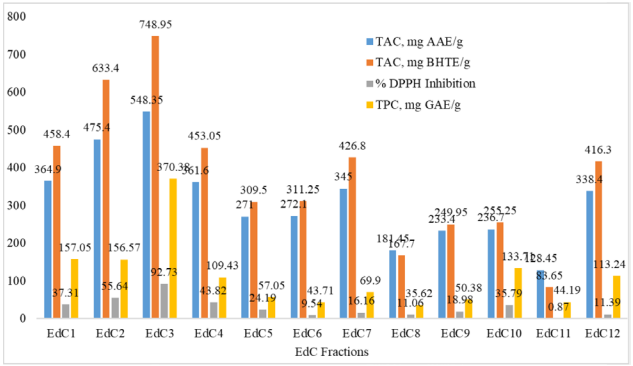

Figure 1: Summary of the *in vitro* antioxidant assay results for EdC fractions.

RESULTS

Bioactivity of the fractions from the chloroform-soluble partition (EdC) of *E. debile*

Fractionation of EdC via silica chromatography yielded 12 fractions which were consequently subjected to BSLA and *in vitro* antioxidant assays.

Brine Shrimp Lethality Assay (BSLA)

Toxicity of EdC fractions were tested at a single dose of 500 mg/L. Percent mortality data for 6 and 24-hr exposure were recorded. Summary of results are presented in Table 1.

In vitro Antioxidant Assays

In vitro antioxidant experiments, i.e. total antioxidant capacity (mg AAE/g and mg BHTE/g), DPPH radical scavenging activity (%DPPH inhibition) and total phenolics content (mg GAE/g) were performed in all EdC fractions. The results are presented in Figure 1.

Structure Elucidation

Among the EdC fractions, EdC3 showed the most potential results and is thus allowed to undergo further

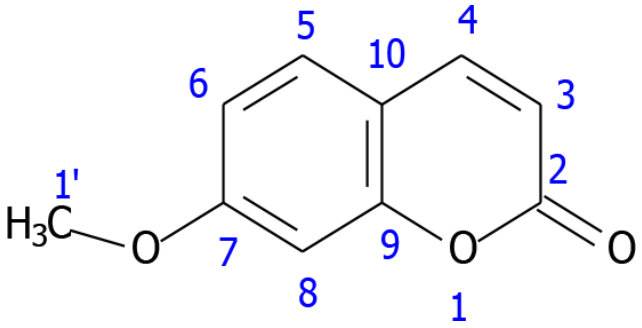

Figure 2: Molecular structure of herniarin (7-methoxycoumarin).

Table 2: ^{1}H and ^{13}C NMR data of herniarin from *E. debile*.

Position	150 MHz ^{13}CNMR in CD_3OD δ_C, ppm	600 MHz ^{1}HNMR in CD_3OD δ_H, ppm (m, J)[a]
2	169.59	-----
3	114.53	6.34 (d, 15.9)
4	145.48	7.62 (d, 15.9)
5	110.26	7.20 (d, 8.1)
6	122.58	7.09 (dd, 8.3 and 1.9)
7	149.09	-----
8	115.05	6.84 (d,1.9)
9	147.95	-----
10	126.39	-----
1'	55.03	3.92 (s)

[a] m (multiplicity), J (coupling constant in Hz)

The key homonuclear ^{1}H-^{1}H (COSY) correlations of herniarin from *E. debile* are shown in Figure 3. On the other hand, the heteronuclear HSQC and HMBC correlations are evident in Figure 4 and 5, respectively.

purification techniques to afford herniarin or 7-methoxy coumarin (Figure 2).

The ^{1}H and ^{13}C NMR signal of herniarin from *E. debile* is presented in Table 2.

DISCUSSION

BSLA

After 6 hrs, 3.33% mortality was observed for EdC2 while no brine shrimp was killed in the other fractions. After 24 hrs, however, EdC1, EdC2 and EdC3 showed significant toxicity. EdC2 gave the highest percentage mortality of 100.0% while EdC3 and EdC2 gave percentage mortality values of 96.67% and 93.33%, respectively.

In vitro Antioxidant Assays

EdC3 consistently gave the highest result in the *in vitro* antioxidant assays. In terms of total antioxidant capacity and total phenolic content, EdC3 gave 548.35 mg AAE/g, 748.95 mg BHTE/g and 370.38mg GAE/g, respectively. All other fractions showed intermediate values which are quite low compared to that of EdC3. For DPPH radical scavenging activity, only EdC3 exhibited a significant inhibition of DPPH radical, which is very comparable to that of ascorbic acid and BHT. EdC3 inhibited 92.73% of the DPPH radicals while the rest of the fractions did not show potential radical scavenging activity. With this, EdC3 was allowed to undergo further separation processes in order to obtain an isolate.

However, it is worth noting that comparison of the bioactivity results obtained for EdC in the previous study[5] with that of fraction EdC3 revealed that higher antioxidant levels and radical scavenging activity were obtained for the fraction. A crude plant extract is a complex mixture of a large array of compounds that may interact antagonistically interfering with or masking the activity of one another. Moreover, majority of active components in crude extract is present at a very low concentration and may not show specific activity.[16] Upon fractionation, however, bioactive compounds may have been separated and concentrated to greater purity.

Structure Elucidation

The herniarin was obtained as colorless needle-like crystals. The structure of herniarin from *E. debile* was elucidated by extensive 1D and 2D NMR. Its LC-MS Total Ion Chromatogram showed one UV (254 nm)-absorbing compound at a retention time of 23.58 min. The molecular ion peaks at m/z 177 [M+H], 194.9 $[M+H+H_2O]^+$ and 208.9 $[M+H+CH_3OH]^+$ are observable in the positive ESI-MS chromatogram. This suggests a compound with a molecular weight of 176 units corresponding to the molecular formula $C_{10}H_8O_3$ of 7-methoxycoumarin or herniarin (Figure 2).[17]

Characteristic ^{1}H and ^{13}C NMR signals (Table 2) of herniarin showed resemblance to a benzopyrone skeleton.[18] Moreover, recorded data are consistent with those reported in the literature.[17]

The ^{1}H NMR spectrum of the herniarin displays six signals. Five of them can be attributed to aromatic hydrogen atoms which give resonances in the 6-8.5 ppm region. The specific chemical shifts for the aromatic protons are: H_4 (7.62), H_5 (7.20), H8 (6.84), H_3 (6.34), H_6 (7.09). A single peak at 3.92 ppm is a signal arising from three protons and was assigned to the methyl protons (H_1), of the methoxy group (-OCH_3). The ^{13}C NMR resonances of herniarin, on the other hand, displays ten carbon signals. The nine signals are typical of a benzopyrone skeleton and the other one was attributed to a carbon in a methoxy (C_1), moiety. Further, the ^{13}C NMR

spectrum shows 5 methine signals at δ_C 145.48 (C_4), 122.58 (C_6), 115.05 (C_8), 114.53 (C_3) and 110.26 (C_5) ppm and 4 quaternary carbon signals at δ_C 169.59 (C_2), 149.09 (C_7), 147.95 (C_9) and 126.39 (C_{10}) ppm. Among the four quaternary carbons, one signal came from an aromatic carbon (C_7) bonded to oxygen atom of a methoxy group while the other is from a carbonyl carbon (C_2). Lastly, the signal at 55.03 ppm was assigned to the carbon ($C_{1'}$) atom of the methoxy group (-OCH_3). The homonuclear COSY spectrum of herniarin shows 3 significant cross peaks. Very long range distance (three bonds or more) proton-proton coupling may be detected, as seen for the coupling of H_6 and H_8 (Figure 3), for samples analyzed at higher frequencies (at least 450 MHz in ^{1}H NMR).[19] On the other hand, the heteronuclear proton-carbon correlations are evident in the HSQC (Figure 4) and HMBC (Figure 5). The HSQC and HMBC spectra are instrumental in detecting the overall skeletal connectivity of the molecule. As can be seen in Figure 4, the direct correlations of C_3 (δ 114.53) and H_3 (δ 6.34), C_4 (δ 145.48) and H_4 (δ 7.62), C_5 (δ 110.26) and H_5 (δ 7.20), C_6 (δ 122.58) and H_6 (δ 7.09),

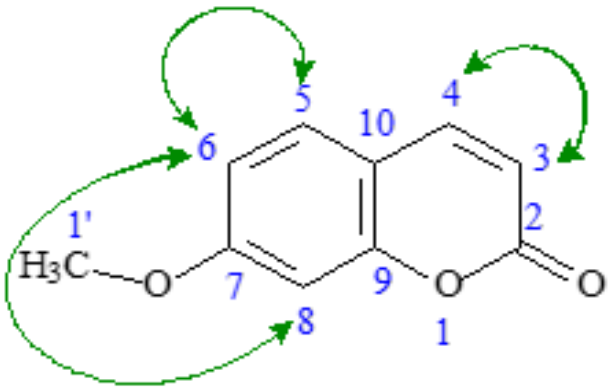

Figure 3: Key COSY correlations in herniarin from *E. debile*.

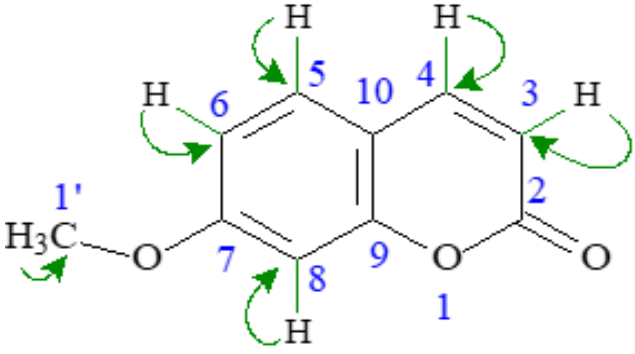

Figure 4: Key HSQC correlations in herniarin from *E. debile*.

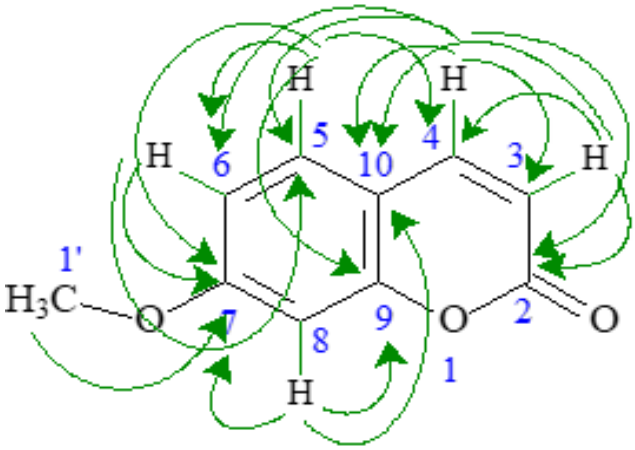

Figure 5: Key HMBC correlations in herniarin from *E. debile*.

C_8 (δ 115.05) and H_8 (δ 6.84) and $C_{1'}$ (δ 55.03) and $H_{1'}$ (δ 3.92) are shown. Moreover, said correlations are further verified with the HMBC which showed long-range (2 to 3-bond distance) heteronuclear couplings of carbon and proton nuclei. In the HMBC, correlation of a non-protonated signal at 169.59 ppm, corresponding to three-bond and two-bond couplings, with two doublet proton signals at 7.62 (H_4) and 6.34 (H_3) ppm, respectively, is observable. This suggests that the said carbon should be assigned to the C_2 (-C=O) position of the coumarin moiety. Another quaternary carbon, δ 149.09, showed four significant cross peaks. The cross peaks represent couplings of carbon to aromatic methine protons H_5, H_6 and H_8 and the methyl protons $H_{1'}$ of the methoxy group. These correlations made possible the assignment of δ 149.09 signal to C_7 (-C-OCH_3) of the coumarin moiety. Lastly, Carbon atoms C_{10} and C_9 are assigned according to the HMBC interactions of H_3, H_4, H_5 and H_8 as well as H_8 and H_5 with the carbon signals at 126.39 and 147.95 ppm, respectively.

CONCLUSION

Toxicity and antioxidant assay-guided isolation of the chloroform-soluble partition of the whole plant sample of *E. debile* yielded herniarin. Thus, *E. debile* can be a potential source of bioactive compounds.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in the subject matter or materials discussed in this manuscript.

ABBREVIATIONS

EdC: Chloroform-soluble partition of *E. debile*; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **TAC:** Total Antioxidant Capacity; **TPC:** Total Phenolics Content; **AAE:** Ascorbic Acid Equivalent; **BHTE:** Butylated hydroxytoluene Equivalent; **GAE:** Gallic acid Equivalent; **COSY:** Homonuclear correlation spectroscopy; **HSQC:** Heteronuclear Single Quantum Coherence Spectroscopy; **HMBC:** Heteronuclear Muliple Bond Correlation Spectroscopy.

SUMMARY

Bioassay-guided isolation of bioactive components in *Equisetum debile* Roxb. was conducted. The plant extracts of *E. debile* were obtained by extraction with 95% ethanol and serial partitioning of the ethanolic extract into hexane-, chloroform- and water-soluble extracts. *In vacuo* concentration of the obtained extracts yielded EdH, EdC and EdA, respectively. Fractionation EdC afforded a bioactive fraction EdC3. EdC3 consistently gave the highest values in total antioxidant capacity, total phenolics content and DPPH radical scavenging activity. Further purification of EdC3 through silica gel column chromatography, washing and recrystallization afforded a colorless needle-like crystal. Extensive 1D and 2D NMR and LC-MS analyses led to the structure elucidation of 7-methoxycoumarin or herniarin with a molecular weight of 176.0 mass unit and a molecular formula of $C_{10}H_8O_3$.

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