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A comparative analysis of antioxidant, anti-inflammatory, and anticancer activities of the crude and fractionated extracts from *Distichochlamys benenica*

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Abstract

Distichochlamys benenica is a newly discovered plant belonging to the *Distichochlamys* family, which exerts many important biological activities. However, the anti-cancer, anti-inflammatory, and anti-oxidant effects of the crude (ME) and fractioned extracts of *D. benenica,* such as hexane (HE), chloroform (CE), and ethyl acetate extracts (EAE), have not been elucidated yet. The antioxidant activity of the extracts was evaluated via DPPH free radical scavenging and phosphomolybdate assays. The anti-inflammatory potential of the extracts was assessed via their inhibitory effects on protein denaturation and nitric oxide production. The anti-cancer effect of the extracts against A549 and MDA-MB-231 cell lines was determined via MTT assay. The results revealed that among four extracts, the polyphenol concentration was highest in EAE (350.59 ± 13.26 mg GAE/g) while the flavonoid content was highest in HE and ME. Furthermore, the greatest DPPH radical capture efficiency was demonstrated by EAE (89.76 ± 0.68%), and total antioxidant activity was greatest in HE (204.18 ± 3.51 mg AAE/g). The greatest suppression of NO generation was similarly shown by EAE and HE, with IC_{50} values ranging from 3.72-4.06 µg/mL, respectively. Additionally, HE and EAE exhibited an inhibitory effect on protein denaturation (ranging from 12.76%-28.81%). HE and EAE had the greatest effectiveness against MDA-MB-231 cell lines in the antitumor test. These findings validate the potent antioxidant, anti-inflammatory, and anti-cancer properties of *D. benenica* extracts, especially EAE and HE, and pave the way for the application of the extracts in the pharmaceutical industry as promising anti-inflammatory and anti-cancer medicines.

Keywords: *Distichochlamys benenica*, fractionated extracts, anti-oxidant activity, anti-inflammatory effect, anti-cancer effect.

Introduction

Free radicals are molecular entities capable of existing independently and containing an unpaired valence electron, many of them are unsteady and highly reactive such as reactive oxygen species (ROS) or reactive nitrogen species (ROS). Suppose the imbalance between free radicals being generated and the body's antioxidant defense system occurs- in that case, oxidative stress arises and leads to damage not only in molecular levels, such as proteins, nucleic acids, and lipids but also in cellular and tissue levels [1]. In addition, both oxidative stress and inflammatory response, the physiological reaction of animals to protect them from infection and tissue injury, have a close relationship and interplay together in the onset of some diseases. The oxidative stress can trigger pro-inflammatory signaling pathways and accumulation of inflammatory mediators, and the inflammation response in turn can promote the overproduction of free radicals [2]. Consequently, oxidative stress plays a significant role in the development of numerous inflammatory-associated diseases, including arthritis, asthma, bronchitis, and inflammatory bowel disease [3]. Furthermore, some previous studies indicate that long-term exposure to oxidative stress can oxidize the molecules of life, trigger inflammatory signaling pathways, alter transcription factors, genes, and proteins, and result in the progression of cancers [4]. According to Vaidya et al. (2020), the induction of some transcription factors such as NF-kB, HIF-1α, and Nrf2 which interplay in inflammation response and oxidative stress lead to the reforming in growth factors, oncogenes, tumor suppressor genes, cell

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cycle regulators, which in turn boosts the cancer development [5]. Therefore, some research has been conducted to target oxidative stress and cancer-promoting inflammation to treat or prevent cancers [6, 7].

Medicinal plants are promising sources for the extraction of bioactive compounds which possess a variety of health-beneficial effects, due to their abundant bioactive compounds and the well-described information from long historical use of these species. Among them, several species of the Zingiberaceae family, such as ginger and turmeric, have a high potential application in the pharmaceutical industry for treating oxidative stress or inflammatory-associated diseases, and cancer [8]. The Zingiberaceae family, also called the ginger family, comprises more than 1960 species and about 57 genera. These species are mainly distributed in either tropical or subtropical parts globally, with considerable populations in Africa, America, Asia, and Southeast Asia [9]. In 1995, M. F. Newman made the initial discovery of the genus Distichochlamys, a new genus of the Zingiberaceae family, in Vietnam National Park named Bach Ma, located in Thua Thien Hue Province [10]. Within this genus, four species have been determined: *D. citrea* [10], *D. orlowii* [11], *D. rubrostriata* [12], and the most recently discovered, *D. benenica* [13]. The extracts or essential oils from some representatives from the Zingiberaceae family are known for their anticancer, antibacterial, antioxidant, and anti-inflammatory potentials [14, 15, 16]. Recent studies have also explored the biological activities of *Distichochlamys* species [17, 18], like anti-oxidant, anti-diabetic, and anti-thrombotic activities, although research on *D. benenica*'s biological activities is still very limited. Most studies focus on its essential oils, including reports on the chemical composition, mosquito larvicidal effects, and antimicrobial properties [19], as well as the chemical profile and acetylcholinesterase inhibitory effects of its rhizome essential oil [20]. Additionally, there is only one article examining the antibacterial and anti-inflammatory activities of compounds isolated from *D. benenica* [21]. To date, there have been no studies on the biological activities of different fractions extracted from *D. benenica*, which suggests that the scientific medical potential of *D. benenica* has not been fully exploited. In this work, we prepared the crude (methanol, ME) and different fractionated extracts from *D. benenica* including hexane (HE), chloroform (CE), and ethyl acetate extracts (EAE), determined and compared their antioxidant contents, such as polyphenols and flavonoids, as well as their bioactivities, like antioxidant, anti-inflammatory and anti-cancer potentials for investigation of their potential utilization in the pharmaceutical industry.

Materials-Methods

2.1. Chemicals and reagents

Bovine serum albumin at molecular biology grade was obtained from Himedia Laboratories company (Maharashtra, India). The Griess reagent was purchased from Promega (WI, USA), and the Folin-Ciocalteu reagent was obtained from Merck (Darmstadt, Germany). Media and fetal bovine serum were provided by Cytiva (MA, USA). Dimethyl sulfoxide (DMSO), dexamethasone, and lipopolysaccharide (LPS) were purchased from MilliporeSigma (MA, USA). All extraction solvents (methanol, chloroform, n-hexane, and ethyl acetate) were at laboratory reagent grade from Chemsol company (Ho Chi Minh City, Vietnam), and all reagents used in this study were at analytical levels instead of the others would be stated.

2.2. Preparation of the crude and fractionated extracts

Distichochlamys benenica was cultivated in Phu Vinh commune, A Luoi rural district, Thua Thien Hue Province, Vietnam. The samples were collected in October 2023 and authenticated by morphological comparison with voucher specimens from the herbarium at the Faculty of Chemistry, University of Education, Hue University [21]. After harvesting, the raw materials were washed and unused parts were removed. Then the rhizomes of *D. benenica* were dried, ground, and extracted by soaking in methanol. Specifically, 315 g of dried *D. benenica* was extracted with 2.0 L of methanol, repeating the extraction 4 times, each time lasting for 5 days. Next, a rotary vacuum evaporator (at 60°C, 190 mBar) was used to eliminate the solvent and obtain the methanol extract (ME) (Figure 1).

A part of the methanol extract (ME) of *Distichochlamys benenica* was re-dissolved in distilled water at a ratio of 1 extract: 10 water (w/v). Firstly, the suspension was transferred to a separating funnel and partitioned with n-hexane (ratio 1 extract: 2 hexane, v/v). The mixture was thoroughly shaken then let it settle to separate the methanol and hexane. The upper layer of hexane was collected, and then the procedure was repeated the partitioned extraction with hexane more 2 times. The resulting extracts were combined and the residue solvent was removed using a rotary vacuum evaporator (at 60°C, 190 mBar) until the hexane extract (HE) was in solid form.

Following fractional extraction with hexane, the remaining liquid portion was extracted once more using a chloroform solvent (ratio 1 extract: 2 chloroform). To separate the extract and chloroform, the mixture was shaken and then let it settle. After that, the bottom layer of chloroform was collected and the partitioned extraction with chloroform was repeated two more times. The resulting extracts were combined and the residue solvent was removed using a rotary vacuum evaporator (at 60°C, 190 mBar) until the chloroform extract (CE) was in solid form.

Ethyl acetate was mixed with the remaining liquid after the chloroform extraction process (ratio 1 extract: 2 ethyl acetate). Layers of ethyl acetate and water were separated by shaking the mixture thoroughly and let them settle. The top layer of ethyl acetate was collected and the procedure was repeated 2 more times. The resulting extracts were combined and the residue solvent was removed using a rotary vacuum evaporator (at 60°C, 190 mBar) until the ethyl acetate extract (EAE) was in solid form. The extracts (ME, HE, CE, and EAE) were divided into several vials, and a free-drying procedure was applied to obtain the extract powders, then the powders were stored at

Figure 1. Preparation of *D. benenica* extracts. A. Fresh sample of *D. benenica*; B. Rhizome after drying and grinding; C. Dried rhizome soaked in methanol solvent; D. Methanol extract of *D. benenica*.

4°C until use in further experiments.

2.3. Determination of total flavonoid content (TFC)

The total flavonoid content in the extracts was determined through a colorimetric assay with aluminum chloride [22]. To each test tube, 0.5 mL of extracts at a concentration of 5 mg/mL was added, along with 1.5 mL of methanol. Subsequently, 0.1 mL of 1 M potassium acetate together with 0.1 mL of 10% $AlCl_3$ were added. Distilled water was then added to reach a final volume of 5.0 mL. Absorbance was quantified at 415 nm using a spectrophotometer. The TFCs of the extracts were calculated using a quercetin calibration curve and the results were presented as milligrams of quercetin equivalents (QE) per gram of extract (mg QE/g).

2.4. Determination of total polyphenol content (TPC)

The total polyphenol content of the extracts was determined by the Folin-Ciocalteu method [23]. Specifically, 1600 µL of the extracts was put together with 200 µL of Folin-Ciocalteu reagent. After a 3-minute incubation, 200 µL of 20% (w/v) sodium carbonate was added. Following thorough mixing, the mixture was kept in the dark at room temperature for 30 minutes. Absorbance was quantified at the wavelength of 760 nm using a spectrophotometer. A standard curve was created using gallic acid at concentrations ranging from 0.195 µg/mL to 50 µg/mL. The total phenol content was demonstrated as milligrams of gallic acid equivalents (GAE) per gram of extract (mg GAE/g).

2.5. Antioxidant assay

2.5.1. 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

Following the Tran et al. approach (2020) [24], the antioxidant property of the extracts was evaluated via assessment of the scavenging DPPH (2,2-diphenyl-1-picrylhydrazyl) radical capacity of the extracts. A volume of the diluted extracts (0.1 mL) was combined with 1.5 mL of 0.1 mM DPPH solution (dissolved in methanol). The mixtures were shaken thoroughly and allowed to stand at room temperature for 30 minutes. Absorbance was quantified at 517 nm using a spectrophotometer, with ascorbic acid as a positive control. The free radical scavenging activity percentage was defined using the following formula:

$$\%\, I = (1 - \frac{A_2}{A_1}) \times 100\ (\%)$$

Where A1 represents the control sample absorbance at 517 nm
A2 represents the test sample absorbance at 517 nm

2.5.2. Total antioxidant capacity (TAC) assay

The total antioxidant capacity (TAC) was determined using the protocol described by Khiya et al. (2021) [25]. A reagent solution containing sulfuric acid 0.6 mol/L, ammonium molybdate 4 mmol/L, and sodium phosphate 28 mmol/L was diluted to 1.8 mL, to which 0.2 mL of the extract was put in. After incubating the mixture at 95°C for 90 minutes, it was cooled back to room temperature. The samples were then put in a centrifuge at 5000 rpm for 5 minutes to gain the supernatant. Absorbance was quantified at 695 nm using a spectrophotometer. Ascorbic acid, in concentrations ranging from 37.5 to 600 µg/mL, was applied to create the standard curve. The extract's antioxidant property was demonstrated as milligrams of ascorbic acid equivalents (AAE) per gram of extract (mg AAE/g).

2.6. Cell culture

Human lung cancer (A549), human metastatic breast cancer (MDA-MB-231), and murine macrophage (RAW 264.7)

cell lines were obtained from the cell culture collection of Laboratory of Pharmacology, Faculty of Pharmacy, Ton Duc Thang University, Vietnam. These cells were grown in a suitable environment (DMEM or MEM) supplemented with 10% heat-inactivated FBS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. The cultures were maintained at 37°C in a 5% CO_2 humidified incubator for 2-3 passages before using in further experiments.

2.7. Anti-inflammation assay

2.7.1. Bovine Serum Albumin (BSA) denaturation inhibition assay

With a few minor adjustments, the Tran et al. technique was used to evaluate the *D. benenica* extracts' capacity to suppress albumin denaturation [26]. Briefly, 0.2 mL of a 1% BSA solution was mixed with 0.2 mL per extract (final concentration of 0.725, 015, and 0.3 mg/mL). A volume of phosphate-buffered saline (PBS, pH 6.4, 0.6 mL) was added to this mixture. After 20 minutes of incubation at 37°C, the solution was heated for 10 minutes to 70°C. At 660 nm, the absorbance was measured upon cooling. The positive control was diclofenac sodium (Voltaren, 75 mg/3mL, Lek Pharmaceuticals D.D., Slovenia), while the negative control was a reaction solution without a sample or extract. Using the following formula, the percentage of protein denaturation inhibition (I) was determined:

$$\% I = (1 - \frac{A_2}{A_1}) \times 100\ (\%)$$

Where A1 represented the control sample absorbance at 660 nm; A2 represented the test sample absorbance at 660 nm.

2.7.2 Determining nitric oxide (NO) production inhibition assay

The extract's anti-inflammatory properties were assessed through the measurement of nitric oxide generation using Griess reagent [27]. The murine macrophage cells (RAW 264.7) were seeded in a 96-well plate with a cell density of 1.0×10^4 cells/well, and the cells were grown for 24 hours. After that, the cells were treated for two hours with 4 doses (6.75, 12.5, 25, and 50 µg/mL) of each *D. benenica* extracts (HE, EAE, ME, CE). A vehicle control treated with the same volume of DMSO was included (concentration 0 µg/mL). A positive control was provided by adding dexamethasone (final concentration at 1.96 µg/mL). Subsequently, cells were stimulated for 24 hours using 2 µL of LPS at 0.1 mg/mL. After adding Griess reagent to the culture media in a 1:1 ratio, the mixture was allowed to sit at room temperature for 10 minutes. Using a microplate reader (Infinite 50, Tecan, Switzerland), the absorbance was quantified at 540 nm. A standard calibration curve generated with sequential dilutions of sodium nitrite was used to calculate the nitrite content in each sample. The macrophages' cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide MTT test (Invitrogen) [27]. Another group without adding either LPS or the extracts was used as the control for cell viability. The results of three replicates were used to determine and represent the inhibition of nitric oxide production and cell viability.

2.8. Anti-cancer assay

Applying the MTT test, the extract's anticancer properties on human lung cancer (A549) and breast cancer (MDA-MB-231) cells were accessed [27]. Briefly, A549 and MDA-MB-231 cells were grown for 24 hours after being seeded in 96-well plates at densities of 0.8×10^4 and 0.4×10^4 cells/well, respectively. After that, the medium was switched out for new ones including the extracts (dissolved in DMSO) at a final concentration of 0 (the vehicle control, the same volume of DMSO), 12.5, 25, 50, and 100 µg/mL or doxorubicin (0.58 or 2.90 µg/mL) as a positive control. The cultures were then allowed to incubate with a tested compound or reference drug (doxorubicin, LC Laboratories, MA, USA) for 48 hours. After incubation, the cells underwent four hours of incubation at 37°C in a 5% CO_2 humidified environment with MTT solution (5 mg/mL in phosphate-buffered saline). The generated formazan crystals were dissolved by adding 100 µL DMSO, and a microplate reader (Infinite 50, Tecan, Switzerland) was used to detect the absorbance at 540 nm. The average percentage from three replicates was used to detect the extract's cytotoxicity against A549 and MDA-MB-231 cells.

2.9. Statistical analysis

Each determination was performed three times to ensure accuracy. The experimental findings were presented as the mean ± standard deviation (SD) of three replicates. Statistical examination was conducted using GraphPad Prism 8.0 software. One-Way Analysis of Variance (ANOVA) was applied to evaluate differences across the different treatment groups, and multiple comparisons using the Tukey Honestly Significant Difference post hoc test were then employed. For determining statistical significance, a p-value of less than 0.05 ($p < 0.05$) was used.

Results

3.1. Extract yields

After being extracted with MeOH at room temperature, the crude methanol extract (ME) went through a segmented shake process with hexane, chloroform, and ethyl acetate to form different fractions, namely hexane (HE), chloroform (CE), and ethyl acetate extracts (EAE). The yields of the extracts in this single extraction are shown in Table 1 below. The yield was calculated by dividing the mass of the extract by the mass of the initial dry material. The extraction yield ranged between 11.01% for ME and 0.54% for HE.

3.2. Determination of total flavonoid (TFC) and total polyphenol contents (TPC)

Figure 2 presents the results of the TFC and TPC of the crude and fractionated extracts. Among four extracts, the EAE extract possessed the highest value of TPC with 350.59 ± 13.26

Table 1. Extraction yields of *D. benenica* extracts.

Samples	Mass of the extracts (g)	Yields (%)
ME	34.68	11.01
HE	1.70	0.54
CE	13.87	4.40
EAE	2.26	0.72

ME: methanol extract, HE: hexane extract, CE: chloroform extract, EAE: ethyl acetate extract.

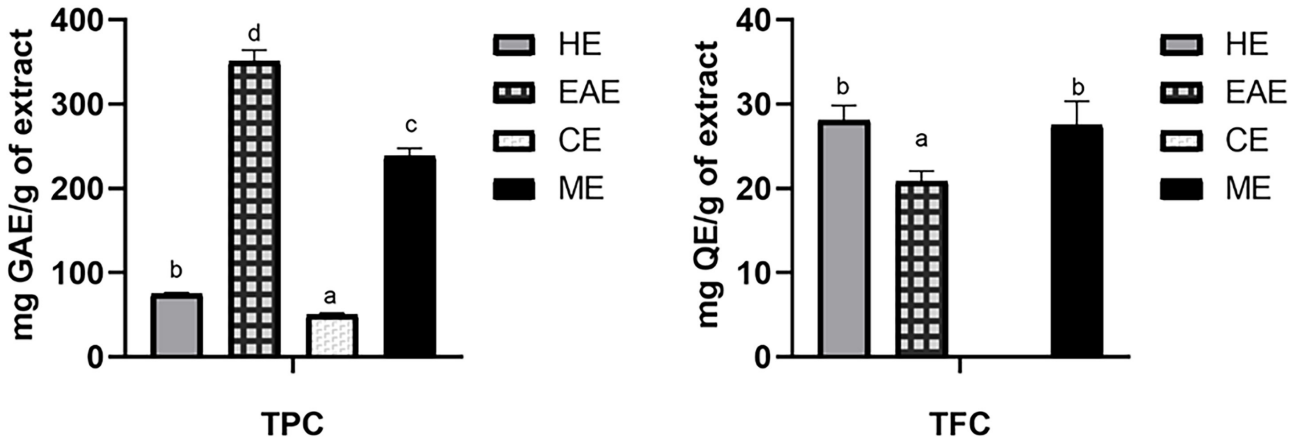

Figure 2. Total polyphenol contents and total flavonoid contents in the *D. benenica* extracts. ME: methanol extract, HE: hexane extract, CE: chloroform extract, EAE: ethyl acetate extract. The letters a, b, c, and d: Results showing statistical significance between the tested groups ($p < 0.05$, using One-way ANOVA and Tukey HSD test). TPC: total polyphenol contents; TFC: total flavonoid contents

mg GAE/g, followed by ME, HE, and CE (238.39 ± 9.15, 74.70 ± 1.15, and 49.72 ± 2.40 mg GAE/g, respectively, $p<0.05$). Furthermore, the TFC of HE was comparable with that of ME and both of them were higher than that of EAE, while TFC was not found in CE. Polyphenols and flavonoids are two well-known groups of antioxidants, the high abundance of polyphenols and flavonoids in the extracts implied the anti-oxidant activity of these extracts. Therefore, we conducted subsequently the DPPH free radical scavenging and phosphomolybdate assays to verify the antioxidant activity of the extracts.

3.3. Antioxidant activity of the extracts

The DPPH assay is widely employed to assess the compound's capacity to function as a scavenger of free radicals or donor of hydrogen. In this method, the decolorization of the violet solution serves as an indication to evaluate antioxidant activity. At a concentration of 5 mg/ml, the DPPH inhibition rate order of the tested extracts is as follows: EAE > ME > HE > CE (Figure 3). Of note, the order of antioxidant activity of the extracts detected via DPPH assay was in line with the polyphenol contents in the extracts. This could be due to the nature of polyphenol as a good scavenger, giving electrons or hydrogen atoms [28]. Figure 3 also provides the total antioxidant capacity of the different extracts as investigated by the total antioxidant capacity method. The highest and lowest total antioxidant capacity was recorded in HE and CE, which were 204.18 ± 3.51 and 29.24 ± 0.60 mg AAE/g of extract respectively. Meanwhile, the remaining TAC value ranged from 81.38 ± 0.53 (ME) to 75.38 ± 1.01 (EAE) mg AAE/g of extract.

3.4. Anti-inflammation activity

3.4.1. Determining nitric oxide (NO) production inhibition assay

The reduction of nitric oxide (NO) generation in RAW 264.7 cells was used to measure the anti-inflammatory impact of *D. benenica* extract. As shown in Table 2, ME and CE extracts showed little toxicity to macrophages at the highest tested dose (50 µg/mL), with cell viability still exceeding 85%, while the cell viability of HE and EAE extracts only above 85% in the lower doses (25 µg/mL) (Table 2). Therefore, the values of inflammatory effects of HE and EAE extracts at the dose of 50 µg/mL were excluded from the calculation of IC_{50}. With IC_{50} values of 3.72 ± 0.41 µg/mL and 4.06 ± 0.49 µg/mL, the EAE and HE showed a strong capacity to control the generation of NO. While the IC50 value for CE could not be obtained due to its exceeding the test concentration (IC50 > 50 µg/mL In addition, the IC_{50} values of NO production inhibition of HE and EAE were approximately 2 times the effective dose of the reference drug (dexamethasone, 1.96 µg/mL), which implied the strong anti-inflammatory effect of the extract. The reference drug

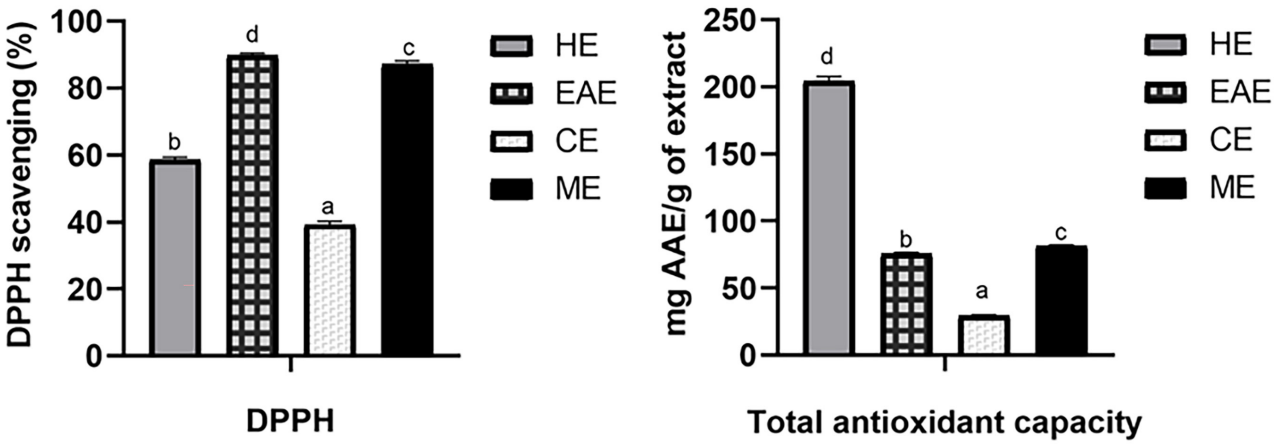

Figure 3. Antioxidant activity of *D. benenica* extracts ME: methanol extract, HE: hexane extract, CE: chloroform extract, EAE: ethyl acetate extract. The letters a, b, c, and d: Results showing statistical significance between the tested groups in the same column ($p < 0.05$).

Table 2. Anti-inflammatory activity of *D. benenica* extracts using NO production inhibition assay.

Sample	NO production inhibition (%)	Cell viability (%)
Dexamethasone (1.96 µg/mL)	45.57 ± 3.12	107.21 ± 1.33
	IC_{50} value for NO production inhibition (µg/mL)	**Cell viability (%)**
HE	4.06 ± 0.49[a]	101.86 ± 1.45 (at the dose of 25 µg/mL)
EAE	3.72 ± 0.41[a]	85.25 ± 3.14 (at the dose of 25 µg/mL)
CE	> 50	99.09 ± 1.71 (at the dose of 50 µg/mL)
ME	10.92 ± 0.60[b]	90.88 ± 6.29 (at the dose of 50 µg/mL)

ME: methanol extract, HE: hexane extract, CE: chloroform extract, EAE: ethyl acetate extract. The letters a, b, c, and d: Results showing statistical significance between the tested groups in the same column ($p < 0.05$, using One-way ANOVA and Tukey HSD test)

was a pure and validated compound, while the extracts were combinations of several compounds, which in turn resulted in a higher concentration of the extracts to achieve the same effectiveness as the reference drug. That finding also suggested further investigation to isolate the main effective compounds from the HE and EAE extracts.

3.4.2. Bovine Serum Albumin (BSA) denaturation inhibition assay

According to Figure 4, EAE had the most anti-inflammatory action when used at 0.3 mg/mL, and its inhibition rate of protein denaturation reached 28.81 ± 0.36%. Of note, the EAE at the dose of 0.3 mg/mL exhibited similar efficacy with diclofenac at the same dose (p>0.05). At 0.3 mg/mL, the inhibitory effect of EAE on protein denaturation was higher than that of HE (20.52 ± 0.88, p<0.0001). On the contrary, at the studied doses, neither CE nor ME showed the capacity to suppress protein denaturation. Furthermore, the inhibitory effects on protein denaturation of EAE and HE have increased in a dose-dependent manner, which suggests EAE and HE could be good candidates for the development of anti-inflammatory agents.

3.5. Anti-cancer assay

The MTT test was used to assess the anticancer effects of *D. benenica* extracts. Even though all the extracts showed anti-cancer effects, only the HE and EAE could determine the IC_{50}

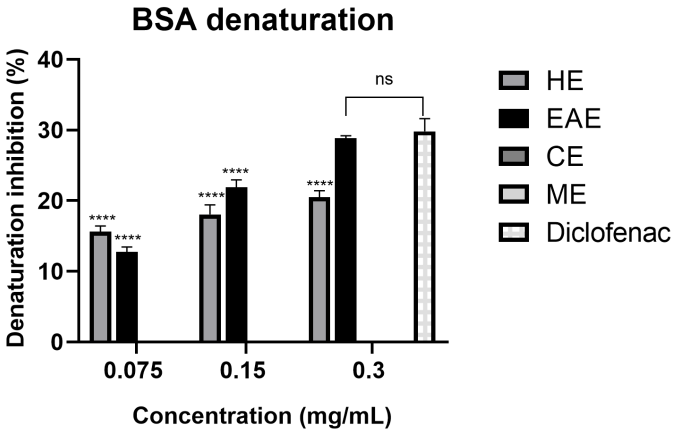

Figure 4. Anti-inflammatory activity of D. benenica extracts using BSA denaturation inhibition assay. ME: methanol extract, HE: hexane extract, CE: chloroform extract, EAE: ethyl acetate extract. The mark **** ($p < 0.0001$) denoted statistical significance among the tested groups with positive control, diclofenac (using One-way ANOVA and Tukey HSD test), the letter ns indicated that there was no statistical significance between the tested group versus the positive control, diclofenac.

Table 3. Anti-cancer activity of D. benenica extracts

Samples	Inhibition rate on A549 cell proliferation (%)	Inhibition rate on MDA-MB-231 cells (%)
Doxorubicin	67.77 ± 5.02 (at a dose of 0.58 µg/mL)	66.06 ± 0.32 (at a dose of 2.9 µg/mL)
	IC_{50} for inhibition of A549 cell proliferation (µg/mL)	**IC_{50} for inhibition of MDA-MB-231 cell proliferation (µg/mL)**
HE	> 100	48.26 ± 2.74^{ns}
EAE	> 100	46.09 ± 2.47^{ns}
CE	> 100	> 100
ME	> 100	> 100

ME: methanol extract, HE: hexane extract, CE: chloroform extract, EAE: ethyl acetate extract. The letter ns indicated that there was no statistical significance between IC_{50} values of the tested groups, HE and EAE ($p > 0.05$, using multiple t-tests)

values (48.26 ± 2.74 and 46.09 ± 2.47 µg/mL) in the MDA-MB-231 cell line (Table 3). As shown in Figure 5, both HE and EAE exhibited the most effective treatment for A549 cancer cell lines at the dose of 100 µg/mL (50.29 ± 2.54% and 48.01 ± 2.23%, respectively). Compared to the positive control, doxorubicin, which showed inhibition rates of 66.06 ± 0.32% for MDA-MB-231 cells (at a dose of 2.9 µg/mL), both EAE and HE showed higher rates of inhibition at the 100 µg/mL ($p<0.001$, Table 3 and Figure 5). At the highest dose of treatment (100 µg/mL), the inhibitory effect of ME on A549 cells was 27.56 ± 1.38*% while CE showed a lower potential inhibition rate (17.56 ± 2.35, $p<0.01$). Moreover, ME also exhibited a higher anti-cancer effect in MDA-MB-231 than that of CE at the dose of 100 µg/mL (43.73 ± 2.84 versus 16.03 ± 1.69%, respectively, $p<0.0001$). Therefore, the potential anti-cancer effect of the extracts followed the order: EAE and HE>ME>CE. This first study revealed the anti-cancer effects of the crude and fractionated extracts from *D. benenica*, which highlighted the further application of these extracts as anti-cancer medicines.

Discussion

Polyphenols and flavonoids are two common anti-oxidants in plants, both of them possess a variety of pharmacological properties such as anti-diabetic, anti-cancer, anti-osteoporotic, anti-obesity, anti-oxidant, cardio-protective, neuro-protective, and anti-inflammatory effects [29, 30]. However, there is no study reporting polyphenol and flavonoid contents in the crude and fractionated extracts of *D. benenica*. The presence of these compounds in the extracts has been confirmed in this study, which gives a possibility of health-beneficial effects of the extracts including antioxidant, anti-inflammatory, and anti-cancer effects. In the previous study, the presence of flavonoids in other species of the *Distichochlamys* genus, like *D. citrea,* has also been recorded. Still, the total flavonoid concentration has not been studied yet [18]. Moreover, Tung et al. (2023) reported that the polyphenol content of 6.02 -12.81% of fractionated extracts of *D. orlowii*, another member of the *Distichochlamys* genus. That is consistent with our results in which the polyphenols of the extracts range from 49.72 –

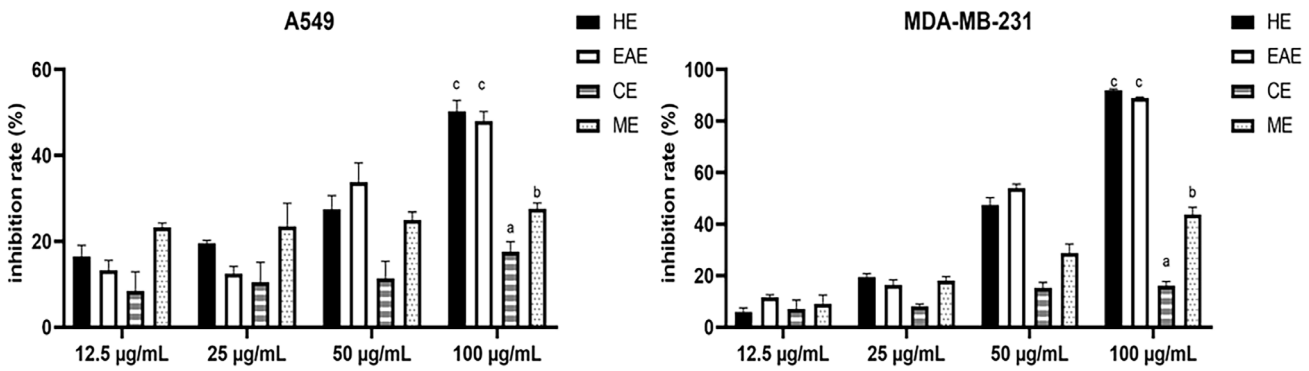

Figure 5. Inhibitory effects of *D. benenica* extracts against A549 and MDA-MB-231 cell proliferation.ME: methanol extract, HE: hexane extract, CE: chloroform extract, EAE: ethyl acetate extract. The statistical differences among groups were determined using One-way ANOVA and multiple comparisons (Tukey HSD test). The letters a, b, c, and d - Results show statistical significance among the extracts treated at the concentration of 100 µg/mL ($p < 0.05$).

350.59 mg GAE/g. Of note, TPCs of ME and EAE extracts are higher than that of the methanol extract of *D. orlowii* [31], which suggests these extracts of *D. benenica* as rich sources for polyphenol extraction. The finding also suggests *D. benenica* as a promising source to extract and isolate bioactive compounds for the pharmaceutical industry, especially in polyphenols. According to Van Chen et al., some flavonoids or polyphenols, such as 5-O-caffeoylquinic acid, kaempferol, and 5-hydroxy-3,7,4'-trimethoxyflavone, may account for the anti-oxidant activity of *D. citrea* rhizome extract [32]. In this study, the DPPH radical scavenging capacities of the extracts of *D. benenica* correlate with the total polyphenol contents, in which both the top two polyphenol-rich extracts (EAE and ME) also rank first and second in scavenging DPPH radical potential. In the previous study, Van Chen et al. (2022) indicated that the ethyl acetate fraction was also the most anti-oxidant activity, which was similar to our findings. Furthermore, the data from Nguyen et al. (2023) also supported our results, in which the methanol extract exhibited the strongest DPPH radical scavenging capacity and a great amount in TPC [17]. In addition, Nguyen et al. (2024) also observed that methanol and ethyl acetate were two suitable solvents for obtaining the high polyphenol and anti-oxidant activity extracts from guava leaves [33]. According to Brglez Mojzer et al. (2016), methanol is a sufficient solvent to extract low molecular weight polyphenols, which explains the high abundance of TPC in the methanol extract of *D. benenica* [34]. On the other hand, HE has the most impressive total anti-oxidant capacity 204.18 ± 3.51 mg AAE/g, followed by those of ME, EAE, and CE. Of note, HE and ME also possess the highest TFC, followed by EAE and CE (not detected). Although there is no direct correlation between TPC and TFC of the extracts with their anti-oxidant activity in both DPPH and phosphomolybdate assays, the most abundant TPC and TFC extracts (EAE and HE) also are the most powerful extracts in DPPH scavenging or total anti-oxidant capacities. This is the first study revealing the anti-oxidant contents and properties in the crude and fractionated extracts of *D. benenica*. Among the four extracts, EAE and HE not only possessed the highest inhibitory effect against NO production but also exhibited the anti-inflammatory effect in the prevention of BSA denaturation. Even though some studies have focused on the bioactivities of the *Distichochlamys* genus, especially in *D. benenica*, they have only investigated the bioactivities of essential oil or some isolated compounds of *D. benenica* [19, 20, 21]. For example, Huong et al. have indicated the essential oil of *D. benenica* as a promising mosquito larvicidal and anti-microbial agent [19]. Moreover, Hoang et al. also suggested the essential oil of *D. benenica* for combating neurodegenerative diseases, such as Alzheimer's disease via its potential to inhibit acetylcholinesterase [20]. In a previous study, Pham et al. not only reported the anti-microbial activity of three compounds of *D. benenica* including borneol, trans-o-coumaric acid, and trans-cinnamic acid but also proved the anti-inflammatory effect of the isolated compound (trans-o-coumaric acid) in edema model. This study has proven the anti-inflammatory effects of the crude and fractionated extracts of *D. benenica* for the first time, especially in the prevention of NO production and BSA denaturation. Polyphenol has a modulatory effect on inflammation response via altering the pro-inflammatory cytokine production, inflammatory-related signaling pathways (NF-κB, MAPK, and arachidonic acid), and immune cell regulation [35]. Flavonoids also are potential anti-inflammatory agents that affect a variety of mechanisms from the alteration of IL17 receptor signaling to the management of pro-inflammatory mediators, like prostaglandins or histamine, chemokines, and cytokines [36]. Of note, trans-o-coumaric

acid, a phenolic compound found in the hexan extract of *D. benenica*, is an effective medicine in lessening the severity of edema development via inhibition of the COX-2 pathway [21]. Therefore, the abundance of flavonoids and polyphenols in the fractionated extracts (HE and EAE) may partially contribute to the anti-inflammatory effect of these extracts. According to Yu et al. (2022), free radicals, such as ROS, facilitate macrophage differentiation and cytokine release [37]. Therefore, the strong free radical scavenging effects of these extracts are also linked with their anti-inflammatory effects. Taken together, these findings imply that EAE and HE are promising anti-inflammatory remedies and may be further applied for the treatment of inflammatory illnesses.

All *D. benenica* fractioned extracts can stop the growth of cancer cells with a variety of effectiveness (Figure 5). In the overview, the anti-cancer effects against both cancer cell lines (A549 and MDA-MB-231) of HE and EAE are the highest among the four extracts, followed by ME, and CE (Figure 5). Even though numerous studies have investigated the anti-cancer effect of the species of the Zingiberaceae family, such as ginger or turmeric, a few studies are researching the anti-cancer effect of the *Distichochlamys* genus [14, 38, 39]. In the previous study, 1,8 cineole, a major active compound in *D. benenica* essential oil, has been proven its anti-tumor effect against colon cancer cells, which also gives a possibility of anti-cancer effect of natural compounds or extracts derived from *D. benenica* [20, 40]. This study is the first study reporting on the anti-cancer activity of *D. benenica* crude and fractionated extracts. According to Mir et al. (2020), flavonoids can inhibit cancer cell growth via initiating apoptosis or autophagy or reduce metastasis through altering voltage-gated sodium channels, signaling pathways, for example, NF-κB pathway, and cell migration or invasion [41]. Moreover, polyphenols also can manage cancer progress via reduction of cancer cell proliferation, arresting cell cycle, and alteration of cancer cells' angiogenesis, differentiation, as well as metastasis [42, 43]. The high values of TFC and TPC in HE and EAE of *D. benenica* explain the strong anti-cancer effect of these extracts against both cancer cell lines.

In this study, some health-beneficial effects of the extracts of *D. benenica,* including antioxidant, anti-inflammatory, and anti-cancer effects have been proven for the first time. As mentioned above, there are only four members of the *Distichochlamys* genus. Among them, *D. citrea* is the most well-known species which has been used in folk medicine. According to Nguyen et al. (2022), *D. citrea* leaves can be used as a remedy for healing oxidative stress-associated and cardiovascular diseases [17]. Moreover, some documents also propose the utilization of *D. citrea* to treat infectious diseases and diabetes [32, 44]. The essential oil of *D. orlowii*, another member of the *Distichochlamys* genus, has been suggested to prevent carbohydrate digestion and melanogenesis [45]. The research on the medical application of *D. benenica* is only limited in reporting the anti-acetylcholinesterase, anti-microbial, and anti-inflammatory effects of its essential oil or isolated compounds [20, 21]. These findings are in line with other literature for contributing to the ethno-pharmacological application of species of the *Distichochlamys* genus.

Conclusion

In this study, the presence of polyphenols and flavonoids, two well-known anti-oxidants, in the crude and fractionated extracts of *D. benenica* has been elucidated. Furthermore, all the extracts exhibited anti-oxidant, anti-inflammatory, and anti-cancer effects with a variety of effectiveness. In the scavenging activity, EAE is the most potential extract, while HE is the best for total antioxidant capacity. Among four extracts, EAE and HE are the two most promising extracts for anti-inflammation activities, not only in the suppression of NO production in LPS-induced macrophages (IC_{50} values ranging from 3.72-4.06 μg/mL) but also in the prevention of bovine serum albumin denaturation assay. These extracts are also good candidates for the treatment of breast cancer, with IC_{50} values against the MDA-MB-231 cell line ranging from 46.09-48.26 μg/mL. In conclusion, this study not only confirms the value of biological compounds contained in the *D. benenica* extracts, such as polyphenols and flavonoids, for the first time but also opens up many opportunities for future medical applications of *D. benenica* extracts, particularly EAE and HE, in combating cancers, oxidative stress, and inflammation associated disorders.

Conflict of Interest

The authors declare no conflict of interest.

Author contributions

Experimental conceptualization and design: Tran GB; methodology: Tran GB, Pham TV, Do BH; investigation: Tran GB, Pham TV, Tran TXT, Do BH, data collection and handling: Tran GB, Do DT, Tran TXT; formal analysis and interpretation: Tran GB, Do BH, Tran TXT, project administration and funding acquisition: Tran GB; validation: Tran GB, Pham VT; draft manuscript: Tran GB, Tran TXT, Do BH, Pham VT, Do DT; review & editing: Tran GB.

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