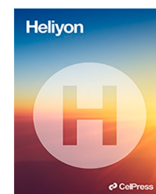
Heliyon 10 (2024) e36686

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Comprehensive phytochemical profiling and biological activities of *Hodgsonia heteroclita* subsp. *indochinensis* seed extracts

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ARTICLE INFO

Keywords:
Antioxidant activity
Antibacterial activity
Antiproliferative activity
Anti-inflammation activity
Seed extract of *Hodgsonia heteroclita* subsp *Indochinensis*

ABSTRACT

Hodgsonia heteroclita subsp. *indochinensis*, a member of the Cucurbitaceae family, is utilized in traditional medicinal remedies based on indigenous wisdom. This study aimed to comprehensively identify and analyze the bioactive phytoconstituents within *H. heteroclita* subsp. *indochinensis* seeds. Seeds were sequentially extracted with n-hexane, ethyl acetate, and methanol. Liquid chromatography-mass spectrometry analysis detected ferulic acid, salicylic acid, cucurbitacin E, stigmasterol glucoside, and β-sitosterol glucoside in all extracts. The total phenolic content in the HH(S)-EtOAc and HH(S)-MeOH was 14.22 ± 1.58 and 12.98 ± 1.03 mg gallic acid equivalent/g, respectively. Consequently, the HH(S)-EtOAc demonstrated antioxidant activity with an IC_{50} of 1.10 ± 0.28 mg/mL, while the HH(S)-MeOH displayed strong antioxidant potential with an IC_{50} of 0.04 ± 0.00 mg/mL according to an ABTS assay. Antibacterial evaluations of both the HH(S)-hexane and HH(S)-EtOAc revealed significant activity against *Staphylococcus aureus* (zone of inhibition (ZOI): 13.67 ± 2.31 and 11.67 ± 1.53 mm, respectively) but limited activity against *Escherichia coli* (ZOI: 7.33 ± 0.58 and 7.67 ± 0.58 mm, respectively). Additionally, the extracts exhibited low minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values, ranging from 62.50 to 250 mg/mL. The antiproliferative activity of seed extracts was assessed against two breast cancer cell lines (MCF-7 and MDA-MB-231), normal breast cells (MCF10A), and human embryonic kidney (HEK) 293T cells, through MTT and clonogenic assays. The results revealed IC_{50} values exceeding 400 μg/mL, indicating that the extracts are safe. Furthermore, all seed extracts (50 μg/mL) exhibited potent anti-inflammatory activity, evident by their substantial inhibition of nitric oxide production ($p < 0.001$) and *inducible nitric oxide synthase* (*iNOS*) gene expression ($p < 0.05$) in LPS-induced RAW264.7. These findings demonstrate the potential for *H. heteroclita* subsp. *indochinensis* seed

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https://doi.org/10.1016/j.heliyon.2024.e36686
Received 30 January 2024; Received in revised form 28 May 2024; Accepted 20 August 2024
Available online 27 August 2024
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extracts in the development of functional foods, nutraceuticals, and dietary supplements due to their diverse bioactive compounds and substantial biological activities, particularly their anti-inflammatory effects.

1. Introduction

Natural plant products are increasingly used in the pharmaceutical, health food, therapeutic drug, and cosmetic industries due to their notable biological activities [1,2]. Many plant products that have high concentrations of phytoconstituents, including triterpenoids, phytosterols, polyphenols, flavonoids, and phenolic acids, have been traditionally used to prevent and treat various diseases and illnesses [3]. Various plant parts, including flowers, leaves, fruits, stems, and roots, contain bioactive compounds with diverse functions, including antibacterial, antifungal, antioxidant, antiproliferative, and anti-inflammatory properties [4]. According to the World Health Organization (WHO), plants possessing therapeutic potential or serving as precursors to pharmaceutical drugs are classified as medicinal plants. Numerous medicinal plants are utilized as source materials for the synthesis of drugs in the pharmaceutical industry. Currently, there is a growing global inclination towards effective herbal medicines due to their biological activities with minimal side effects [5].

Hodgsonia heteroclita subsp. *indochinensis*, commonly known as "Making" in Thai, belongs to the Cucurbitaceae family. It is a rare and endangered species that is actively conserved by the Plant Genetic Conservation Project, initiated by Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG). *Hodgsonia heteroclita* subsp. *indochinensis* has been discovered in elevated terrains (400–1500 m above sea level) across South Asia, including regions in Thailand, Myanmar, Laos, Cambodia, Bangladesh, and India [6]. Various components of this plant, including its leaves, stems, pulp, and seeds, have been employed as traditional medicinal remedies based on indigenous wisdom. Additionally, the fruit pulp of *H. heteroclita* is traditionally used as an antidiabetic medicine in India. A previous study reported that the ethanol extract of fresh fruit of *H. heteroclita* showed strong antioxidant activity, which was related to the presence of phytoconstituents such as flavonoids, phenolics, alkaloids, saponins, and steroids. A heavy metal analysis showed that the extract contained high levels of iron, chromium, and copper, while levels of the toxic metals cadmium and lead were within the acceptable range [7]. Moreover, the edible seed contains lipids, proteins, carbohydrates, minerals, vitamin E, and other phytoconstituents [8]. Nevertheless, the phytoconstituents found in the seed extracts of this plant and their associated biological activities remain unclear.

In this study, *H. heteroclita* subsp. *indochinensis* seeds were sequentially extracted using n-hexane, ethyl acetate, and methanol. The bioactive phytoconstituents within seed extracts were determined by liquid chromatography-mass spectrometry (LC-MS) analysis. In addition, the biological activities of the seed extracts were evaluated, including the antioxidant, antibacterial, antiproliferative, and anti-inflammation activities.

2. Materials and methods

2.1. Plant materials

Hodgsonia heteroclita subsp. *indochinensis* fruit was collected from forests near Mae Sai Pa Meing village, Long Khod Sub-district, Phrao District, Chiang Mai Province, Thailand, in December 2022. Authentication and identification of plant samples were conducted by Assistant Professor Dr. Boonchuang Boonsuk from the School of Science, University of Phayao (Phayao, Thailand). The sample plant was deposited in Queen Sirikit Botanic Garden Herbarium, The Botanical Garden Organization, Ministry of Natural Resources and Environment, Thailand, under the reference number QBG No. 140208.

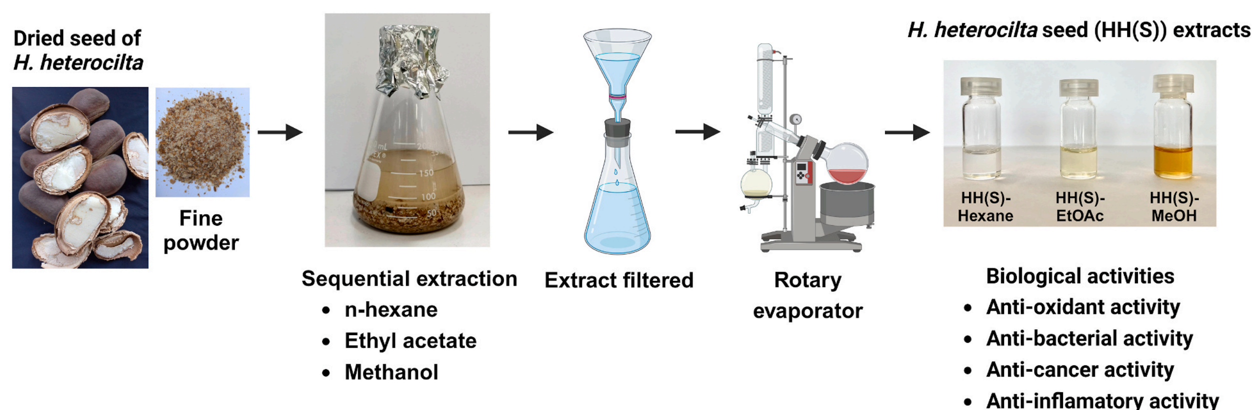

Fig. 1. Illustrates the preparation process for *H. heteroclita* seed extract.

2.2. Preparation of seed extracts

The *H. heteroclita* subsp. *indochinensis* seeds (350 g) were washed with deionized water and air-dried at room temperature to remove any excess moisture, a crucial step to prevent the growth of mold and bacteria during the extraction process. Subsequently, the dried seeds were ground into a fine powder. The powdered seeds were sequentially extracted using three solvents with increasing polarity, n-hexane (hexane), ethyl acetate (EtOAc), and methanol (MeOH). This sequential extraction targeted a broad range of compounds. Each solvent extraction was carried out for 24 h to maximize the yield of targeted compounds. The extraction process was repeated three times to ensure a thorough extraction. Following each extraction, the mixture was filtered to remove any solid material. The filtered solutions were then evaporated to dryness under reduced pressure at 40–45 °C to obtain the seed extracts (Fig. 1).

2.3. Phytoconstituent profiling using LC-MS

The seed extracts (10 mg) were dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted with methanol to a concentration of 200 μg/mL. The diluted samples were filtered through a syringe filter with a 0.2-μm nylon membrane. The phytoconstituent profile of all seed extracts was analyzed using an Agilent Technologies 1290 infinite liquid chromatography (LC) instrument coupled to an Agilent 6540 series QTOF-MS equipped with an ESI source, a Diode Array Detector (DAD) (Agilent Technologies, Santa Clara, USA), and Agilent Poroshell 120 EC-C18 (4.6 × 150 mm, 2.7 μm). The separation involved a chromatographic gradient (0.1 % formic acid in water and 0.1 % formic acid in acetonitrile) for 33 min at 35 °C, with a 200 μL/min flow rate.

2.4. Free radical scavenging activity analysis using an ABTS assay

An ABTS assay was conducted as described previously [9]. Briefly, 190 μL of ABTS working solution was mixed with 10 μL of sample and then placed in the dark at room temperature for 10 min. The absorbance was then measured at 734 nm using a microplate reader (Cytation 3, Biotek instrument). A standard curve was established using standard Trolox, and the results are presented as Trolox equivalent per gram dry weight (μmol TE/g DW).

2.5. Free radical scavenging activity analysis using a DPPH assay

A DPPH assay was performed as described previously [10], with some modifications. Briefly, 22 μL of sample was mixed with 198 μL of 150 μM DPPH reagent. The mixture was immediately shaken and incubated in dark at room temperature for 30 min. The absorbance was measured at 520 nm using a microplate reader (Cytation 3, Biotek instrument). Trolox was used as the standard, and the results were present as Trolox equivalents per gram dry weight (μmol TE/g DW).

2.6. Total phenolic content (TPC) determination

The total phenolic contents (TPC) were determined using Folin-Ciocalteu method. In brief, 20 μL of various concentrations of samples were mixed with 100 μL of 10 % Folin-Ciocalteu reagents and 80 μL of 7.5 % sodium carbonate. The mixture was then incubated at room temperature for 15 min. The absorbance values of samples were read using microplate reader (Cytation 3, Biotek instrument) at OD760. The values of TPC were expressed in milligrams of gallic acid equivalents per gram extract of a sample (mg GAE/g extract).

2.7. Agar disc diffusion method

The antibacterial activity of the seed extracts was examined against Gram-negative (*Escherichia coli* TISTR1984) and Gram-positive (*Staphylococcus aureus* TISTR746) bacteria using the disk diffusion technique as described by Kamilla et al. (2009) [11]. Reference bacterial strains were obtained from the Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani, Thailand. The bacterial culture was added to a 0.85 % normal saline solution to achieve turbidity equivalent to the McFarland 0.5 standard (1.5×10^8 CFU/mL). Bacterial solutions were swabbed onto Mueller–Hinton agar (MHA; HiMedia Laboratories Pvt. Ltd., Mumbai, India, M173) plates using a sterile cotton swab.

The HH(S)-hexane, HH(S)- EtOAc, HH(S)- MeOH extracts (500 mg/mL) were applied to the sterile paper disk. Chloramphenicol (an antibiotic), and dimethyl sulfoxide (DMSO) were utilized as positive and negative controls, respectively. Subsequently, the inoculated plates were incubated at 37 °C for 24 h in an aerobic environment. After incubation, the inhibition zones were measured in millimeters using calipers.

2.8. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay

The MIC of the seed extracts was determined using the microtiter broth dilution method described previously [12]. The MIC assay was conducted in a sterile 96-well plate. Five different concentrations (62.5, 125, 250, 500, and 1000 mg/mL) of each seed extract were prepared in MHB medium, with 50 μL added to each well of the 96-well plate. Subsequently, 50 μL of bacterial suspension containing approximately 1.5×10^5 CFU/mL of *S. aureus* or *E. coli* was added to each well. Chloramphenicol (30 μg/mL) and DMSO served as positive and negative control treatments, respectively. Resazurin solution was added to each well to indicate bacterial growth

inhibition. The 96-well plate was incubated at 37 °C for 24 h before color determination. The MIC value was defined as the lowest concentration of the sample that inhibited bacterial growth, observed as the first clear well.

The MBC assay was performed following the method described by Ozturk et al. (2006) [13]. Ten microliters were collected from each of three wells from the MIC assay: the well showing the MIC value and the next two wells with higher concentrations than the MIC well. These samples were then plated onto MHA medium, and bacterial colonies were observed after 16–20 h of incubation at 37 °C. The lowest concentration of seed extract that killed 99.9 % of the bacterial inoculate was considered the MBC value.

2.9. Cell cultures

Human embryonic kidney (HEK) cells (293T [#CRL-1573; ATCC, Manassas, VA, USA]), normal breast cells (MCF10A [CRL-10317; ATCC]), breast cancer cells (MCF-7 [#HTB-22; ATCC] and MDA-MB-231 [#HTB-26; ATCC]) and mouse macrophages (RAW267.4 [#TIB71; ATCC]) were cultured in Dulbecco's Modified Eagle Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10 % (v/v) fetal bovine serum (FBS; Gibco) and 1 × antibiotic and antimycotic solution (Gibco). The cultures were maintained at 37 °C in a humidified atmosphere with 5 % CO_2.

2.10. MTT assay

The cells were plated in a 96-well plate at a density of 5×10^3 cells/well and incubated for 24 h. Subsequently, the cells were exposed to various concentrations (0–400 μg/mL) of seed extracts or the anticancer drug cisplatin (0–400 μM) and incubated for 24, 48, and 72 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution was added to all test wells and incubated for 2 h at 37 °C. After incubation, the cell culture supernatant was removed, and dimethyl sulfoxide (DMSO) was added to dissolve the dark blue crystals. The solution was thoroughly mixed, and the optical density (OD) was measured at 570 nm using a microplate reader (Cytation 3, Biotek instrument). The percentages of cell viability and IC_{50} values for samples were determined using GraphPad Prism (version 8.0.1; San Diego, CA, USA).

2.11. Clonogenic assay

Cells were seeded in 6-well plates at 2×10^5 cells/well and incubated for 24 h. Subsequently, the cells were incubated with seed extracts (50 μg/mL) or cisplatin (30 μM) for 24 h. Following treatment, the cells were trypsinized, reseeded in 6-well plates at 1×10^3 cells/well, and cultured in a complete medium for 8–10 days. The adherent cells were fixed with cold absolute methanol for 45 min, stained with 0.5 % (w/v) crystal violet at room temperature for 45 min, and excess crystal violet dye was rinsed off with tap water before allowing the plates to air-dry. The cell colonies on each plate were counted using a light microscope and photographed.

2.12. Nitric oxide (NO) production assay

In brief, RAW 264.7 cells were seeded in a 96-well plate at 4×10^4 cells/well and incubated for 24 h. The cells were then pre-treated with 0.5 μM of dexamethasone or 50 μg/mL of seed extracts for 1 h and stimulated with 10 ng/mL LPS for 24 h. Supernatants (50 μL) were collected and mixed with 50 μL of Griess reagent, followed by incubation at room temperature for 10 min. The optical density (OD) was measured at 540 nm using a microplate reader (Cytation 3, Biotek instrument). The percentage of NO production was calculated relative to the LPS treatment alone (set as 100 %).

2.13. qPCR

Total RNA was extracted from treated RAW267.4 cells using TRIzol reagent (Invitrogen, Auckland, New Zealand). Briefly, the RNA was used as a template for cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-rad, Hercules, CA, USA), following the manufacturer's protocol. The cDNA sample was then used in a qPCR reaction with specific primers for *inducible nitric oxide synthase* (*iNOS*) (forward primer: GAGACAGGGAAGTCTGAAGCAC, reverse primer: CCAGCAGTAGTTGCTCCTCTTC) and SensiFAST™ SYBR master mix (Bioline, London, UK). The fold changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to the housekeeping gene *GAPDH* (forward primer: CATCACTGCCACCCAGAAGACTG, reverse primer: ATGCCAGTGAGCTTCCCGTTCAG).

2.14. Statistical analysis

All experiments were independently conducted at least three times to generate datasets. The mean ± standard error of the mean (SEM) was computed for each dataset. Statistical analysis was performed using GraphPad Prism Software (version 8.0.1). One-way analysis of variance (ANOVA) followed by Tukey's test was employed to determine the significance of differences in the data. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Extraction yield and total phenolic content of seed extracts

Hodgsonia heteroclita subsp. *indochinensis* seeds were extracted with different solvents, which were chosen based on their polarity: hexane is non-polar, ethyl acetate is moderately polar, and methanol is polar. The HH(S)-hexane demonstrated the highest yield (7.4 %), while the lowest yield was observed for the HH(S)-EtOAc (0.63 %). The TPC in the HH(S)-hexane, HH(S)-EtOAc, and HH(S)-MeOH was 7.50 ± 0.61, 14.22 ± 1.58, and 12.98 ± 1.03 mg GAE/g extract, respectively (Table 1).

3.2. Phytoconstituent profiling of seed extracts

Liquid chromatography-mass spectrometry (LC-MS) was used to identify the phytoconstituents in the seed extracts. The retention times, masses, *m/z* (expected)2, chemical formulas, and activities are summarized in Table 2. The HH(S)-hexane contained nine phytoconstituents: 55.56 % triterpenoids, 22.22 % phenolic compounds, and 22.22 % sterols. Similarly, the HH(S)-EtOAc contained nine phytoconstituents: 33.33 % triterpenoids, 33.33 % phenolic compounds, 22.22 % sterols, and 11.11 % amino acids. The HH(S)-MeOH contained 13 phytoconstituents: 38.46 % triterpenoids, 23.08 % phenolic compounds, 15.38 % sterols, 11.11 % amino acids, and 7.69 % flavonoids (Fig. 2A–C and Table 2). The phytoconstituents had diverse functions, including antioxidant, antimicrobial, antiproliferative, and anti-inflammatory activities. Cucurbitacin E, a triterpenoid, was the most abundant phytoconstituent in all seed extracts, followed by the phenolic compounds ferulic acid and salicylic acid (Fig. 2D). Notably, our analysis detected stigmasterol glucoside and β-sitosterol glucoside in all seed extracts. Subsequently, the biological activities of the seed extracts, including their antioxidant, antimicrobial, antiproliferative, and anti-inflammation activities, were assessed.

3.3. Antioxidant activity of seed extracts

The free radical scavenging activities of seed extracts and Trolox (used as a positive control) were evaluated using DPPH and ABTS assays (Fig. 3). Trolox, the HH(S)-EtOAc, and the HH(S)-MeOH exhibited significant and dose-dependent scavenging of the DPPH radical ($p < 0.001$) (Fig. 3A, C, 3D). At 1 mg/mL, Trolox, the HH(S)-EtOAc, and the HH(S)-MeOH exhibited inhibition rates of 98.02 ± 0.21 %, 28.12 ± 2.16 %, and 9.67 ± 0.40 %, respectively. Similarly, in the ABTS assay, known for its direct and stable radical generation, Trolox, the HH(S)-EtOAc, and the HH(S)-MeOH demonstrated potent suppression of the ABTS radical in a dose-dependent manner ($p < 0.001$) (Fig. 3E, G, 3H). At 0.5 mg/mL, Trolox, the HH(S)-EtOAc, and the HH(S)-MeOH exhibited inhibition rates of 69.92 ± 3.08 %, 36.72 ± 1.30 %, and 75.54 ± 5.61 %, respectively. Interestingly, the free radical scavenging activity of the HH(S)-EtOAc was consistent across both assays. In contrast, the HH(S)-MeOH displayed antioxidant activity specifically in the ABTS assay, with a higher activity than the HH(S)-EtOAc, indicating variations in the antioxidant potential of the seed extracts under different assay conditions.

3.4. Antibacterial activity of seed extracts

The antibacterial activity of seed extracts and chloramphenicol (CHL; as a positive control) were investigated according to their zone of inhibition, MIC, and MBC values against pathogenic bacterial strains *E. coli* and *S. aureus*. These bacteria are common pathogenic bacteria that pose significant risks to public health and safety globally. They cause foodborne illnesses and hospital-acquired infections and contribute to antibiotic resistance [14]. Consequently, the treatment and prevention of these infections present critical challenges. Furthermore, these two species are widely used in antimicrobial tests with phytoconstituents. The HH(S)-hexane, HH(S)-EtOAc, and CHL demonstrated notable efficacy in suppressing *S. aureus* growth, with zone of inhibition diameters of 13.67 ± 2.31, 11.67 ± 1.53, and 29.75 ± 1.77 mm, respectively (Fig. 4). Similarly, the MIC and MBC values showed that the HH(S)-hexane and HH(S)-EtOAc suppressed *S. aureus* (62.50 ± 0.00 and 125.00 ± 0.00 mg/mL, respectively). However, the HH(S)-hexane and HH(S)-EtOAc had limited effects on *E. coli*, as indicated by inhibition zone diameters of 7.33 ± 0.58 and 7.67 ± 0.58 mm, respectively, whereas CHL showed a larger inhibition zone (29.75 ± 0.35 mm). The MIC values for the HH(S)-hexane and HH(S)-EtOAc against *E. coli* were 125.00 ± 0.00 and 250.00 ± 0.00 mg/mL, respectively, with corresponding MBC values of 250.00 ± 0.00 mg/mL for both extracts. The HH(S)-MeOH exhibited no inhibitory effects on either *S. aureus* or *E. coli* in the agar disc diffusion experiment. The MIC and MBC values for the HH(S)-MeOH against *S. aureus* were 250 ± 0.00 and 500.00 ± 0.00 mg/mL, respectively, and those against *E. coli* were 104.17 ± 36.08 and 208.33 ± 72.17, respectively (Table 3). These findings suggest that the HH(S)-MeOH may have a greater potential

Table 1
Percentage yield (%) and TPC of *H. heteroclita* seed extract samples.

Extract samples	Initial weight (g)	Weight of extract (g)	%Yield	TPC[a]
HH(S)-Hexane	305	22.58	7.4	7.50 ± 0.61[b]
HH(S)-EtOAc	305	1.91	0.63	14.22 ± 1.58[b]
HH(S)-MeOH	305	3.55	1.16	12.98 ± 1.03[b]

[a] mg gallic acid equivalent/gram extract.
[b] Values are expressed as mean ± SEM with three independent experiments (n = 3) for each dataset.

Table 2
List of major phytoconstituents identified from *H. heteroclita* seed extracts including HH(S)-Hexane, HH(S)-EtOAc, HH(S)-MeOH determined by LC-MS.

No.	RT (min)[1]	Mass	*m/z* (Expected)[2]	Chemical formula	Error (ppm)[3]	Identification	Group	Biological activities
1	1.653	141.1121	175.1193	$C_6H_{14}N_4O_2$	2.52	L-arginine	Amino acid	Precursor of nitric oxide [25]
2	1.806	175.0958	176.103	$C_6H_{13}N3O_3$	0.66	L-citruline	Amino acid	Precursor of arginine [26]
3	10.949	472.3549	531.3667	$C_{30}H_{48}O_4$	−0.67	Kuguacin R	Triterpenoid	Anti-inflammatory activity [27]
4	12.017	164.0468	2230606	$C_9H_8O_3$	−3.23	o-coumaric acid	Phenolic compound	Anticancer activity [21]
5	16.46	194.0571	193.0499	$C_{10}H_{10}O_4$	−4.36	Ferulic acid	Phenolic compound	Antioxidant activity [17]
6	17.082	534.3155	579.3195	$C_{30}H_{46}O_8$	−7.06	Cucurbitacin H	Triterpene	Unknown
7	18.809	532.3045	531.2988	$C_{30}H_{44}O_8$	1.67	Cucurbitacin J	Triterpene	Unknown
8	19.085	138.0317	139.0395	$C_7H_6O_3$	−0.1	Salicylic acid	Phenolic compound	Anti-inflammatory and antioxidant activities [18]
9	19.39	302.0436	325.0307	$C_{15}H_{10}O_7$	3.13	Quercetin	Flavonoid	Antioxidant and anti-inflammatory activities [19]
10	20.655	498.2981	521.2925	$C_{30}H_{42}O_6$	−0.11	Cucurbitacin S	Triterpene	Unknown
11	21.608	618.4104	637.4531	$C_{36}H_{58}O_8$	−4.4	Karaviloside XIII	Triterpene	Unknown
12	21.708	4703400	493.3279	$C_{36}H_{58}O_8$	0.76	Karavilagenin D	Triterpene	Anticancer activity [22]
13	21.808	556304	579.2931	$C_{32}H_{44}O_8$	0.67	Cucurbitacin E	Triterpene	Anticancer and anti-inflammation activities [23,28]
14	23.275	170.0212	169.0143	$C_7H_6O_5$	−2.18	Gallic acid	phenolic compound	Antioxidant, anti-inflammatory, antimicrobial, anticancer activities [16]
15	23.457	574.4216	619.4174	$C_{35}H_{58}O_6$	3.05	Stigmasterol glucoside	Sterol	Antioxidant and anti-inflammatory activities [29]
16	24.197	576.4371	621.4346	$C_{35}H_{60}O_6$	−3.36	β-sitosterol glucoside	Sterol	Immunomodulation activity [20]
17	27.508	586.3864	631.39	$C_{35}H_{54}O_7$	−0.89	Momordicine VIII	Triterpene	Unknown
18	27.775	486.3694	545.3831	$C_{31}H_{50}O_4$	−3.11	Momordicine VI	Triterpene	Anticancer activity [24]

to suppress Gram-negative bacteria compared to other extracts. The antibacterial activities of the HH(S)-hexane and HH(S)-EtOAc against Gram-positive bacteria were particularly noteworthy, specifically against *S. aureus*. Differences in the morphological structures of the bacteria may have contributed to their sensitivity to plant extracts.

3.5. Antiproliferative activity of seed extracts

The cytotoxicity of *H. heteroclita* subsp. *indochinensis* seed extracts was evaluated on three cell lines: MCF-7, MDA-MB-231 (breast cancer), MCF10A (normal breast epithelial), and HEK293T (normal kidney) cells. These cells were treated with several concentrations of seed extracts (0–400 μg/mL) and an anticancer drug (0–400 μM cisplatin) for 24, 48, and 72 h, and then cell viability was determined using the MTT assay. The results revealed that cisplatin exhibited high cytotoxicity against normal cells (HEK293T: IC_{50} for 24 h = 62.29 ± 3.10 μM, IC_{50} for 48 h = 11.59 ± 1.30 μM, and IC_{50} for 72 h = 3.25 ± 0.88 μM; and MCF10A: IC_{50} for 24 h = 74.28 ± 3.24 μM, IC_{50} for 48 h = 37.21 ± 2.72 μM, and IC_{50} for 72 h = 26.22 ± 1.66 μM) and cancer cell lines (MCF-7: IC_{50} for 24 h = 73.80 ± 3.24 μM, IC_{50} for 48 h = 41.49 ± 2.80 μM, and IC_{50} for 72 h = 32.89 ± 2.63 μM; and MDA-MB-231: IC_{50} for 24 h = 46.74 ± 2.89 μM, IC_{50} for 48 h = 44.70 ± 2.86 μM, and IC_{50} for 72 h = 33.28 ± 2.64 μM; Fig. 5A, E, 5I, and 5M). In contrast, the seed extracts exhibited no effect on normal cells (HEK293T: $IC_{50} > 400$ μg/mL; MCF10A: $IC_{50} > 400$ μg/mL) or cancer cell lines (MCF-7: $IC_{50} > 400$ μg/mL; MDA-MB-231: $IC_{50} > 400$ μg/mL). These results suggest that *H. heteroclita* subsp. *indochinensis* seed extracts do not demonstrate cytotoxicity in normal or breast cancer cell lines.

Subsequently, the reproductive cell survival of MCF-7 and MDA-MB-231 cells was assessed through a clonogenic cell survival assay. The cells were exposed to seed extract (100 μg/mL) or cisplatin (used as a positive control) for 24 h. Following treatment, the remaining cells were seeded and cultured for 7–10 days. The percentage of colony-forming MCF-7 cells when treated with the HH(S)-hexane, HH(S)-EtOAc, and HH(S)-MeOH was similar to that of untreated control cells (set as 100 %). The percentage of colony-forming cells after treatment with the HH(S)-hexane, HH(S)-EtOAc, and HH(S)-MeOH was 112.5 ± 4.03, 125.3 ± 25.35, and 116.20 ± 26.53, respectively (Fig. 6A and B). Likewise, the percentage of colony-forming MDA-MB-231 cells after treatment with the HH(S)-hexane, HH(S)-EtOAc, and HH(S)-MeOH was 80.82 ± 14.70, 88.29 ± 9.15, and 111.9 ± 0.83, respectively, and these values were not significantly different from those of untreated control cells (Fig. 6A and C). Furthermore, cisplatin significantly decreased colony formation in the MCF-7 and MDA-MB-231 cell lines ($p < 0.05$ and $p < 0.0001$, respectively). These findings suggest that none of the *H. heteroclita* subsp. *indochinensis* seed extracts suppressed the reproductive viability of the breast cancer cell lines MCF-7 and MDA-MB-231.

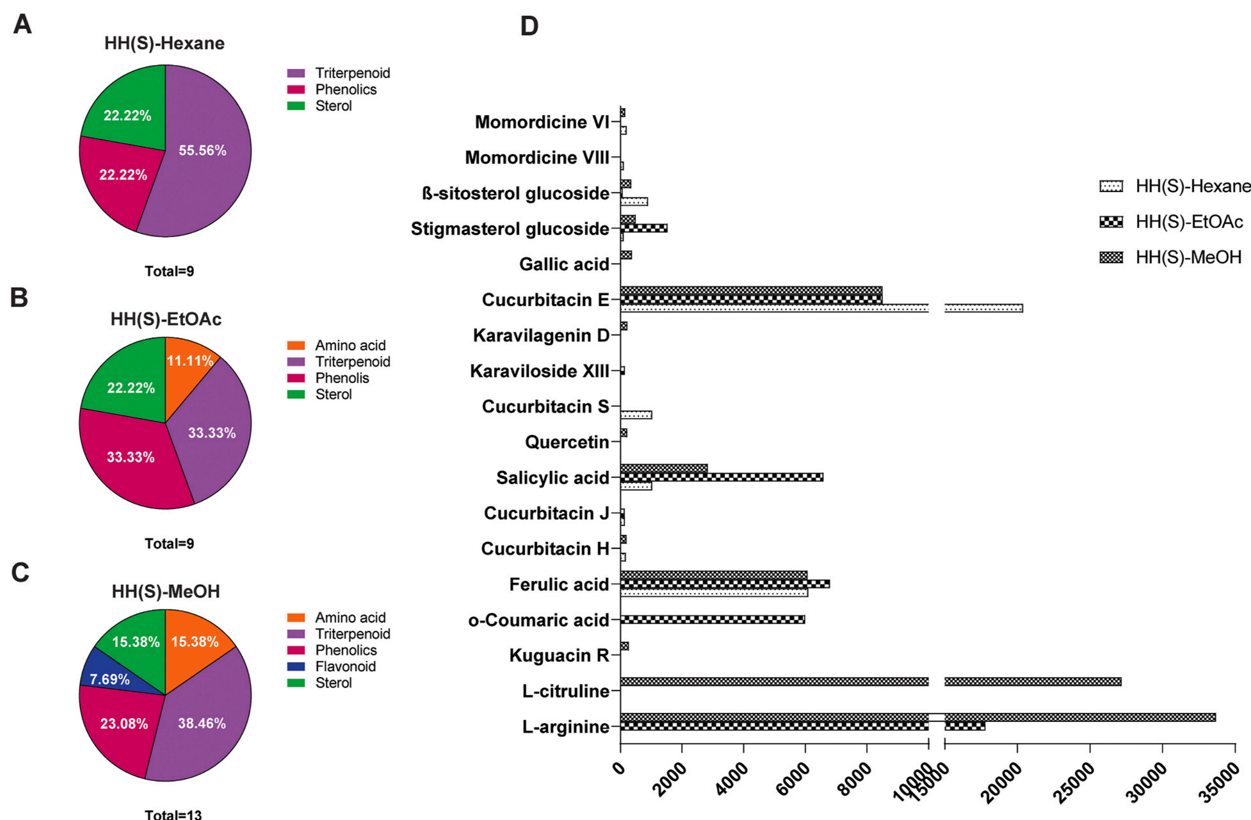

Fig. 2. LC-MS analysis of the phytoconstituents in seed extracts. Pie chart showing the relative distribution of group of phytochemical compounds in each sample including HH(S)-Hexane, HH(S)-EtOAc, HH(S)-MeOH extracts (A–C). The LC-MS were analyzed for the abundances of phytochemicals identified from HH(S)-Hexane, HH(S)-EtOAc, HH(S)-MeOH extracts (D).

3.6. Anti-inflammation activity of seed extracts

The anti-inflammatory potential of seed extracts was evaluated by assessing their impact on the inhibition of NO production and the suppression of *iNOS* gene expression in lipopolysaccharide (LPS)-induced RAW 267.4 cells. Cells were treated with dexamethasone (0–100 μM), LPS (0–100 ng/mL), and seed extracts (0–400 μg/mL) for 24 h. Subsequently, cell viability was assessed using the MTT assay to evaluate the cytotoxicity of these compounds. The cell viability of RAW 267.4 remained unchanged after exposure to dexamethasone at 0–12.50 μM (Supplement Fig. 1A), LPS at 0–12.5 ng/mL (Supplement Fig. 1B), and seed extracts at 0–50 μg/mL (Supplement Fig. 1C), compared to that of untreated cells. Therefore, low doses of dexamethasone (0.5 μM), LPS (10 ng/mL), and seed extracts (50 μg/mL) were used for the subsequent experiment.

Pretreatment of the RAW 267.4 cells with seed extracts or dexamethasone before exposure to LPS significantly lowered the NO levels ($p < 0.0001$) compared to that in cells treated with LPS alone (set at 100 %). The NO production levels following treatment with the HH(S)-hexane, HH(S)-EtOAc, and HH(S)-MeOH and dexamethasone were significantly lower than those without treatment (by 36.30 ± 1.296, 53.33 ± 4.410, 38.33 ± 1.667, and 38.15 ± 3.148, respectively; Fig. 7A). The inhibitory effect of seed extracts and dexamethasone on *iNOS* expression in LPS-induced RAW 264.7 cells were determined using RT–qPCR (Fig. 7B). The fold changes of *iNOS* gene expression relative to cells treated with LPS alone for HH(S)-hexane, HH(S)-EtOAc, and HH(S)-MeOH and dexamethasone treatment were 1.14 ± 0.91 ($p < 0.001$), 1.24 ± 0.23 ($p < 0.01$), 0.84 ± 0.383 ($p < 0.01$), and 0.98 ± 0.11 ($p < 0.001$), respectively.

4. Discussion

Natural plant products are gaining recognition for their positive impact on human well-being and health. Plant products contain a diverse array of phytoconstituents, including phenolics, flavonoids, phytosterols, and terpenes. However, further research is needed to understand the mechanisms by which these products work and to explore potential therapeutic applications. *Hodgsonia heteroclita* subsp. *indochinensis*, known as "Making" in Thai, has been officially designated as a neglected and underutilized crop (NUC) [6]. Despite its traditional cultivation and utilization by local communities, this plant species has received limited attention and investment. In this study, we aimed to identify the phytoconstituents present in *H. heteroclita* subsp. *indochinensis* seed extracts and evaluate their *in vitro* biological activities.

H. heteroclita subsp. *indochinensis* seeds were sequentially extracted using solvents of different polarities: n-hexane (non-polar),

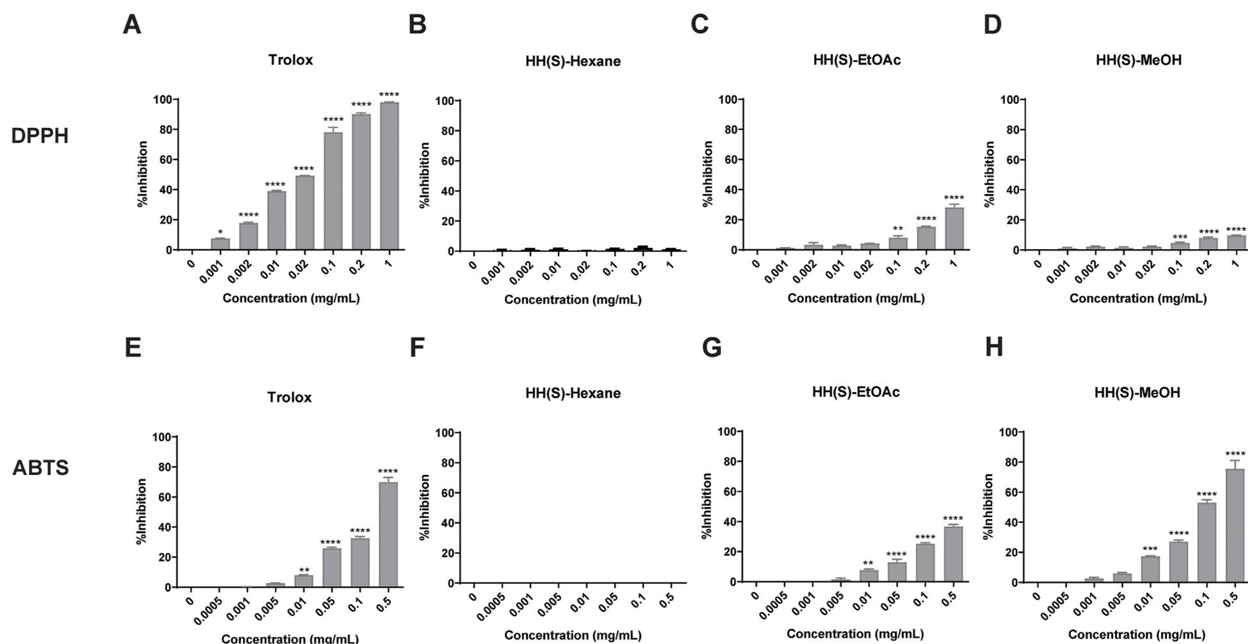

Fig. 3. Antioxidant activity of seed extracts. The radical scavenging capacity of Trolox (used as a positive control) and HH(S) extracts were evaluated using DPPH (A–D) and ABTS (E–H) assays. Values are expressed as mean ± SEM with three independent experiments (n = 3). * ** *$p < 0.0001$, * **$p < 0.001$, * *$p < 0.01$, and * $p < 0.05$ were statistically significant values analyzed by one-way ANOVA compared to the diluent control group.

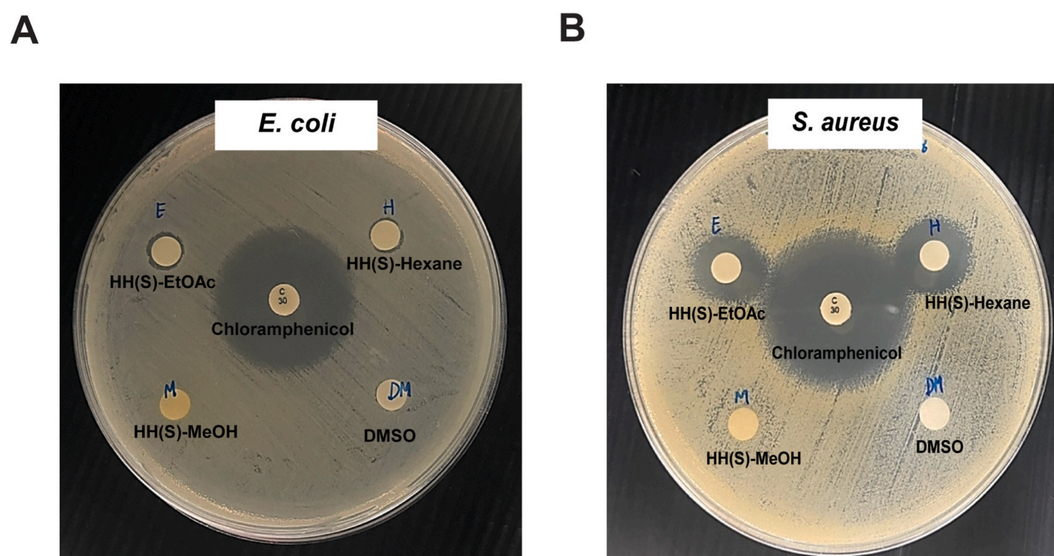

Fig. 4. Effect of seed extracts on bacterial inhibition zones. The 10 μL of 500 mg/mL HH(S)-Hexane, HH(S) -EtOAc, HH(S)-MeOH extracts were tested for inhibition of bacterial growth. The zone of inhibition of HH(S)-Hexane, HH(S) -EtOAc, HH(S)-MeOH extracts, and Chloramphenicol (CHL) against *E. coli* TISTR 1984 (A) and *S. aureus* TISTR 746 (B) were determined after incubation at 37 °C for 24 h.

ethyl acetate (moderately polar), and methanol (polar). The LC-MS method was employed to identify the phytoconstituents in the seed extracts. As illustrated in Fig. 2, cucurbitacin E, a triterpenoid, was primarily found in the HH(S)-hexane, whereas L-arginine, an amino acid, was prevalent in the HH(S)-EtOAc and HH(S)-MeOH, aligning with the polarity of the extraction solvents. Cucurbitacin E, which was present in all extracts, inhibits cancer cell growth and the inflammatory response [15], Among the identified phytoconstituents, gallic acid has also been noted for its antibacterial activity [16]. Additionally, the phenolic compounds, including ferulic acid, salicylic acid, quercetin, and gallic acid, as well as phytosterols, such as stigmasterol glucoside, exhibit antioxidant effects [16–20]. Phenolics (o-coumaric acid and gallic acid) [16,21] and triterpenes (karavilagenin D, cucurbitacin E, and momordicine VI) have anti-cancer

Table 3
The zone of inhibition (ZOI), MIC, and MBC values of HH(S)-Hexane, HH(S) -EtOAc, HH(S)-MeOH extracts on reference bacterial strains.

Samples	*S. aureus* TISTR 746			*E. coli* TISTR 1984		
	ZOI (mm)	MIC (mg/mL)	MBC (mg/mL)	ZOI (mm)	MIC (mg/mL)	MBC (mg/mL)
HH(S)-Hexane	13.67 ± 2.31	62.50 ± 0.00	125.00 ± 0.00	7.33 ± 0.58	125 ± 0.00	250.00 ± 0.00
HH(S)-EtOAc	11.67 ± 1.53	62.50 ± 0.00	125.00 ± 0.00	7.67 ± 0.58	250 ± 0.00	250.00 ± 0.00
HH(S)-MeOH	inactive	250 ± 0.00	500.00 ± 0.00	inactive	104.17 ± 36.08	208.33 ± 72.17
CHL	29.75 ± 1.77	ND	ND	29.75 ± 0.35	ND	ND
DMSO	inactive	inactive	inactive	inactive	inactive	inactive

Values are expressed as mean ± SEM with three independent experiments (n = 3).

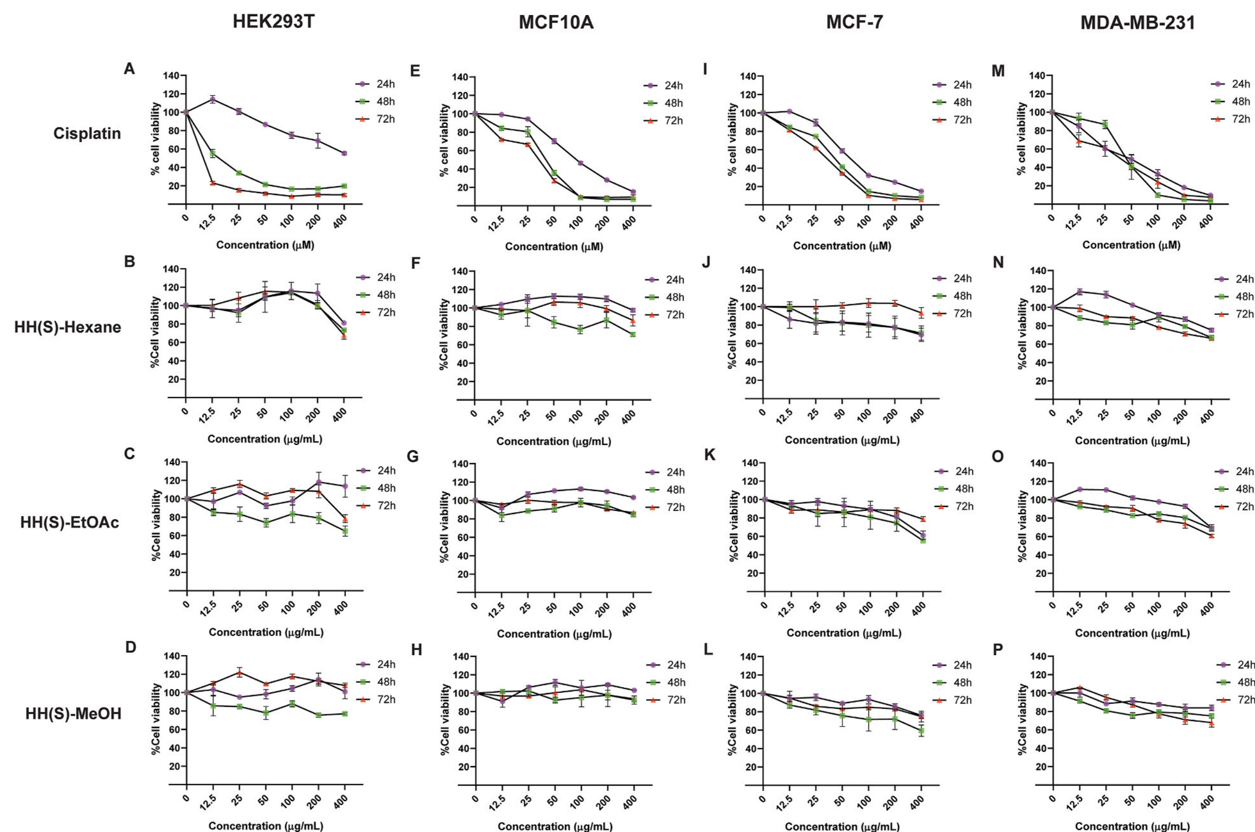

Fig. 5. Effect of seed extracts on cell viability of normal cell lines and breast cancer cell lines. HEK293T (A–D), MCF10A (E–H), MCF-7(I–L), and MDA-MB-231(M–P) cells exposed to HH(S) extracts at concentrations of 0–400 μg/mL for 24, 48, and 72 h. Cell viability was determined by MTT assay. Values are expressed as mean ± SEM with three independent experiments (n = 3).

effects [22–24]. L-arginine and L-citrulline are involved in nitric oxide (NO) production, contributing to the prevention of bacterial growth [25,26]. Various phytoconstituents, including kuguacin R, salicylic acid, quercetin, cucurbitacin E, gallic acid, and stigmasterol glucoside exhibit anti-inflammatory activity [16,18,19,27–29]. However, the biological effects of cucurbitacin H, cucurbitacin J, cucurbitacin S, karavilagenin D, and momordicine VIII remain unclear. All compounds in the seed extracts identified in this study are known for their pharmacological activities (Table 2). In a previous study, LC-MS analysis of an extract from *H. heteroclita* fruit pulp (extracted with 70 % methanol) contained various phytoconstituents, including benzoic, salicylic, o-coumaric, p-coumaric, protocatechuic, caffeic, 2,4-dihydroxybenzoic, gentisic, syringic, gallic, vanillic, and ferulic acids [30]. In the current study, the total phenolic content (TPC; Table 1) and LC-MS analyses, revealed that the HH(S)-EtOAc and HH(S)-MeOH contain four phenolic compounds: ferulic acid and salicylic acid (detected in both extracts), o-coumaric acid (present in the HH(S)-EtOAc), and gallic acid (present in the HH(S)-MeOH). Flavonoids have previously been found in the rind and fruit pulp of *H. heteroclite* [3,31] and flavonoids, phenolics, alkaloids, saponins, and steroids have been identified in ethanol extracts of fresh *H. heteroclita* fruit [7]. Differences in the phytoconstituent compositions and concentrations found in extracts may be attributed to variances in geographical locations, plant cultivation methods, and the specific plant parts used for extraction [32]. Our results show that *H. heteroclita* subsp. *indochinensis* seed extracts contain a range of phytoconstituents, such as phenolics, flavonoids, triterpenes, and sterols, which exhibit diverse biological

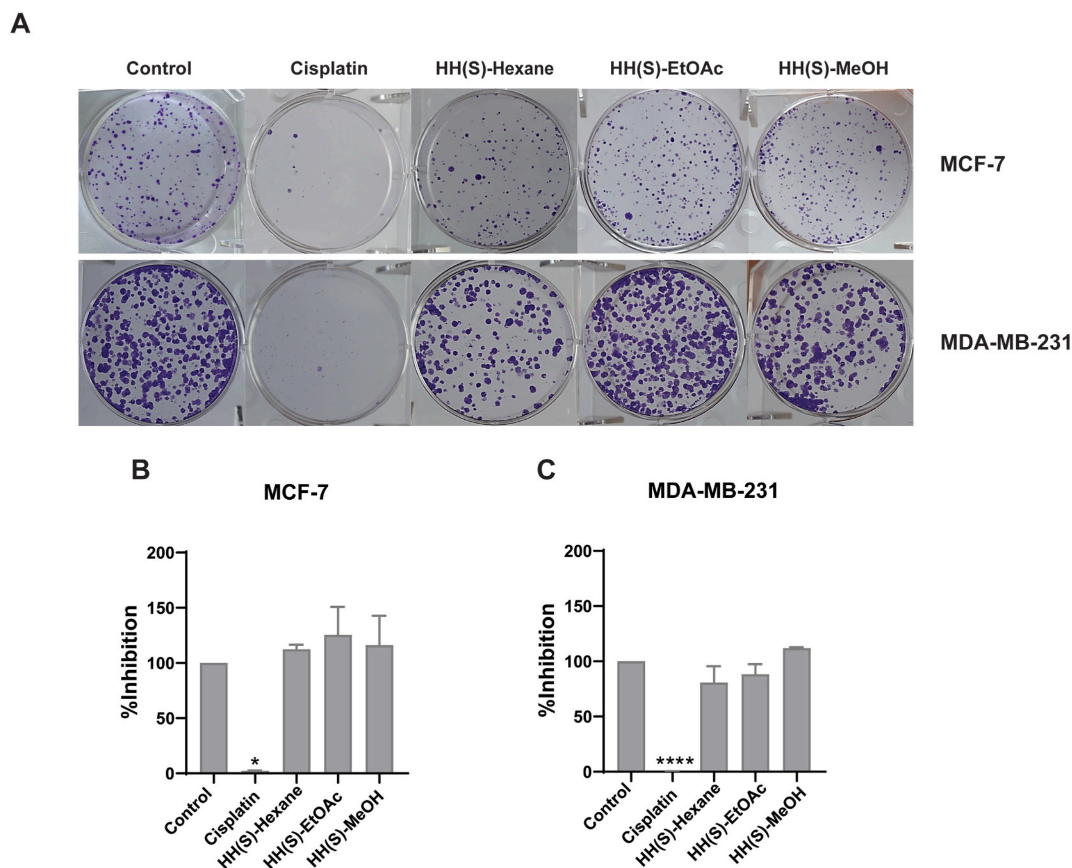

Fig. 6. Effect of seed extracts on the replicative ability of MCF-7 and MDA-MB-231 cells. The MCF-7 (A and B) and MDA-MB-231 (A and C) were exposed to HH(S) extracts (100 μg/mL) or cisplatin (100 μM) for 24 h. The one thousand remaining cells were reseeded for 7–10 days without HH(S) extract or cisplatin treatment. The cell colonies were stained with 0.5 % (w/v) crystal violet and counted under the light microscope. Values are presented as means ± S.E.M. from three independent experiments (N = 3). * ** *$p < 0.0001$ was a statistically significant value analyzed by one-way ANOVA compared to the control group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

activities and warrant further evaluation.

The antioxidant potential of *H. heteroclita* subsp. *indochinensis* seed extracts was assessed using DPPH and ABTS assays, with the HH (S)-EtOAc and HH(S)-MeOH demonstrating notably higher antioxidant activities compared to the HH(S)-hexane. This is likely due to the diverse mixture of compounds present in the HH(S)-EtOAc and HH(S)-MeOH, such as phenolic compounds. Similarly, a methanol extract of *H. heteroclita* fruit pulp demonstrated robust antioxidant activity in both DPPH and ABTS radical scavenging assay [30,33] and an ethanol extract of *H. heteroclita* fruit pulp also demonstrated strong antioxidant activity [7]. Moreover, oilseed cake powder (OCP) extracted from *H. heteroclita* seeds exhibited antioxidant properties and demonstrated potential inhibitory effects against enzymes associated with Alzheimer's disease, such as β-secretase-1 (BACE-1), as well as enzymes linked to hypertension, such as angiotensin-converting enzyme (ACE) [8].

The present study investigated the antimicrobial potential of *H. heteroclita* subsp. *indochinensis* seed extracts against *S. aureus* and *E. coli* using various methods including the agar disc diffusion, MIC, and MBC methods. The HH(S)-hexane and HH(S)-EtOAc exhibited high antimicrobial activity against *S. aureus*, a microorganism responsible for skin and soft tissue infections, while the HH(S)-MeOH showed high activity against *E. coli*, which causes gastrointestinal infections. Additionally, LC-MS analysis revealed the presence of gallic acid in the HH(S)-MeOH, a compound associated with antimicrobial activity [16]. Moreover, both the HH(S)-hexane and HH (S)-EtOAc contained cucurbitacin J, the specific function of which is currently unclear. Cucurbitacins are tetracyclic triterpenes produced by members of the Cucurbitaceae family. Cucurbitacins have various biological activities, including anti-inflammatory, antimicrobial, antioxidant, antiviral, and antihyperglycemic properties [34].

The antiproliferative effects of the seed extracts were assessed on breast cancer cell lines (MCF-7 and MDA-MB-231) as well as normal cell lines (HEK293T and MCF10-A) using MTT and clonogenic assays. The IC_{50} values for the seed extracts against MCF-7, MDA-MB-231, HEK293T, and MCF10-A exceeded 400 μg/mL at 24, 48, and 72 h. Results from the clonogenic assay indicated that the seed extracts did not affect the proliferative capacity of MCF-7 and MDA-MB-231 cells. These findings suggest that *H. heteroclita* subsp. *indochinensis* seed extracts do not possess antiproliferative effects against breast cancer cells. Moreover, the seed extracts

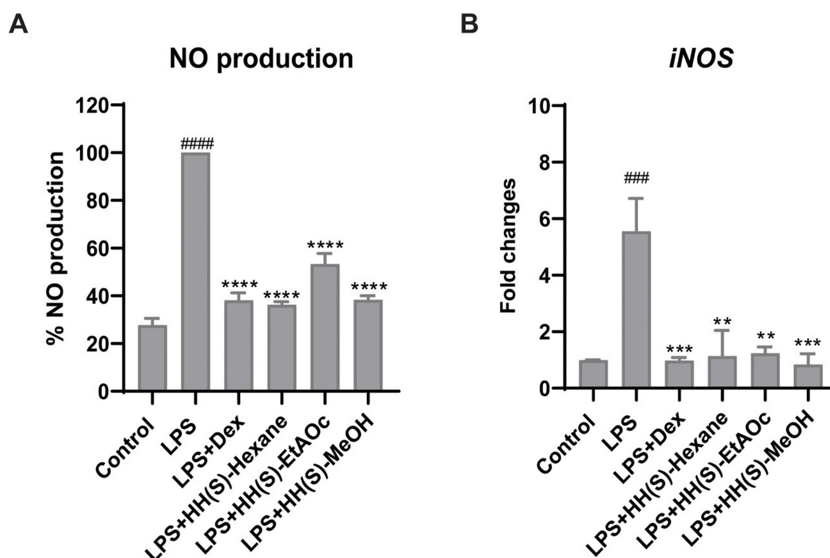

Fig. 7. Effect of seed extracts on NO production and *iNOS* gene expression in mouse macrophage cell lines. RAW264.7 cells were pre-exposed to dexamethasone (0.5 μM) or HH(S) extracts (50 μg/mL) for 1 h and followed treatment with 10 ng/mL LPS for 24 h. Cultured supernatants were collected to determine the NO production using Griess reagent (A). Treated cells were collected to determine the *iNOS* gene expression by qPCR (B). Values are expressed as mean ± SEM with three independent experiments (n = 3). $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ were statistically significant values analyzed by one-way ANOVA compared to LPS treatment alone. $^{\#\#\#\#}p < 0.0001$ and $^{\#\#\#}p < 0.001$ were statistically significant values analyzed by one-way ANOVA compared to the control group.

demonstrated low cytotoxicity to normal cells, implying their safety for use in health and food products. Similarly, *H. heteroclita* oilseed cake powder, comprising 4-hydroxybenzoic acid and ferulic acid as the major bioactive compounds, demonstrated genome safety by not inducing DNA mutations in the Ames test [8].

The anti-inflammatory potential of *H. heteroclita* subsp. *indochinensis* seed extracts was investigated in RAW 267.4 cells induced by LPS. Pre-treatment with the seed extracts significantly reduced the NO levels and *iNOS* expression in the LPS-induced RAW 267.4 cells. These results suggest that the seed extracts have anti-inflammatory potential, likely due to the presence of salicylic acid, cucurbitacin E, stigmasterol glucoside, and β-sitosterol glucoside (Fig. 2). Previous studies have found that salicylic acid derived from willow bark inhibits cyclooxygenase (COX), preventing prostaglandin formation during the inflammation processes in arthritis [35,36]. Moreover, cucurbitacin E has a wide spectrum of pharmacological properties, including anti-tumor [37] and anti-inflammation potential [38]. Furthermore, ß-sitosterol-ß-D-glucoside significantly reduces nitric oxide (NO) production in LPS-induced RAW 264.7 cells, leading to a decrease in the secretion of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β [39]. Stigmasterol inhibits pro-inflammatory and matrix degradation mediators involved in cartilage degradation in osteoarthritis (OA), partly through NF-κB pathway inhibition [40]. These findings contribute to our understanding of the potential mechanism by which *H. heteroclita* subsp. *indochinensis* seed extracts can reduce inflammation.

5. Conclusion

Our experiments revealed that *H. heteroclita* subsp. *indochinensis* seed extracts are rich in a diverse range of phytoconstituents. Depending on the polarity of the extraction solvent used, the seed extracts exhibited distinct antioxidant, antibacterial, and anti-inflammatory properties. The phytoconstituent analysis and subsequent assessment of biological activities indicated the safety and efficacy of the extracts. These results highlight the promising potential of *H. heteroclita* subsp. *indochinensis* seed extracts in the development of functional foods, nutraceuticals, and dietary supplements, positioning them as a preferable and sustainable choice for promoting health and well-being. However, *in vivo* and clinical studies are necessary to confirm the effects of the seed extracts, particularly in terms of their anti-inflammatory properties.

Funding

This research was funded by the Thailand Science Research and Innovation Fund and the University of Phayao (Grant no. FF66-RIM048), the Unit of Excellence of Research and Development for Cancer Therapy at the University of Phayao (Grant no. UoE62015), the Office of the Permanent Secretary, Ministry of Higher Education, Science, Research and Innovation (OPS MHESI), Thailand Science Research and Innovation (Grant no. RGNS 63-145) and partially supported by Chiang Mai University through Aussara Panya.

Declarations on competing interests

The authors declare no conflicts of interest and that the funder played no role in the study design, data collection, data analysis, data interpretation, writing of the manuscript, or the decision to publish the results.

Ethics approval and consent to participate

Not applicable.

Data availability statement

All available data generated by experiments during this study are included in this published article.

CRediT authorship contribution statement

Chutamas Thepmalee: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Krissana Khoothiam:** Methodology, Formal analysis, Data curation. **Natthaphon Thatsanasuwan:** Methodology, Formal analysis, Data curation. **Artitaya Rongjumnong:** Methodology, Formal analysis, Data curation. **Nittiya Suwannasom:** Validation, Investigation. **Chonthida Thephinlap:** Validation, Investigation. **Piyawan Nuntaboon:** Methodology, Formal analysis, Data curation. **Aussara Panya:** Project administration, Funding acquisition. **Orada Chumphukam:** Methodology, Formal analysis, Data curation. **Ratchanaporn Chokchaisiri:** Methodology, Formal analysis, Data curation.

Declaration of competing interest

The authors declare no conflicts of interest and that the funder played no role in the study design, data collection, data analysis, data interpretation, writing of the manuscript, or the decision to publish the results.

Acknowledgements

The authors would like to thank Professor Dr. Pa-thai Yenchitsomanus, Mahidol University, Thailand for providing the HEK293T and MCF-7 cell lines, Assistant Professor. Grissana Pook-In, University of Phayao, Thailand for the MCF10A cell line, Dr. Kamonwan Jongsomchai University of Phayao, Thailand for the MDA-MB-231 cell line, and Assistant Professor Dr. Kanokkarn Phromnoi University of Phayao, Thailand for the RAW267.4 cell line.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36686.

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